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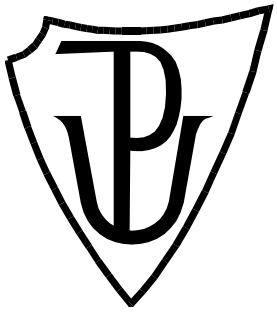

Generation and analysis of new transgenic barley lines expressing recombinant antimicrobial peptides.

Ph.D. THESIS

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I hereby declare that this Ph.D. thesis has been written solely by myself and that all the sources quoted in this work are listed in the “References” section. All published results included in this work are approved by co-authors.

In Olomouc .....

Edita Holásková

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Abstrakt

Znepokojivý nárůst výskytu bakteriálních rezistentních kmenů představuje závažný problém v celosvětovém měřítku, který vyžaduje naléhavé řešení, aby nedošlo k tzv. post-antibiotické éře, kdy by rezistentní bakterie způsobující třeba i jinak "banální infekce" mohly mít opět na svědomí lidské životy. Antimikrobiální peptidy (AMPs) LL-37, beta-defensin 2 a pexiganan představují slibná činidla pro léčbu infekčních onemocnění způsobených rezistentními kmeny mikroorganismů, protože jsou aktivní vůči celé řadě patogenů včetně těch, jež si vyvinuly antibiotickou rezistenci. Navzdory jejich potenciálu je možnost praktického použití AMPs v medicíně limitována, a to především vysokou cenou spojenou s jejich produkcí. Z tohoto hlediska představuje využití rostlin pro jejich produkci velice slibnou strategii, která však vyžaduje řadu optimalizací. Ty jsou v zásadě spojeny s potenciální toxicitou AMPs vůči hostitelské rostlině, malým množstvím vyprodukovaného peptidu a případně také s problémy spojenými s izolací produktu a jeho biologickou aktivitou. Ve snaze vyřešit výše uvedená omezení bylo vyselektováno několik fúzních peptidických či proteinových translokačních, stabilizačních a purifikačních sekvencí a byl testován jejich vliv na množství LL-37 přechodně akumulovaného v listech tabáku. Výsledky této analýzy poskytly hodnotná data, která byla zohledněna při návrhu konstruktů pro heterologní expresi LL-37 v ječmeni.

Následně byly metodou stabilní transformace připraveny a analyzovány fertilní linie ječmene exprimující buď pod konstitutivním, anebo vybraným zrnově specifickým promotorem různé varianty fúzních *AMP* genů podrobených kodonové optimalizaci pro ječmen. Přestože byla na proteinové úrovni metodou imunolokalizace s využitím specifických protilátek potvrzena přítomnost všech navržených AMPs v endospermu ječmenného zrna, nebylo možné metodou Western blotu detekovat rekombinantní pexiganan ani lidský beta-defensin 2. Naproti tomu Western blot analýza transgenních zrn ječmene exprimujících lidský LL-37 potvrdila akumulaci peptidu, která dosahovala hodnoty až 0.55 ug rekombinantního peptidu na gram zrna. Bylo prokázáno, že použití zrnově specifického promotoru je spojeno s dosažením větších výtěžků, než je tomu v případě konstitutivního promotoru, dále že fúze LL-37 k proteinu maltózu-vázajícímu (MBP) zvyšuje jeho stabilitu v desikovaném zrnu a že použití enterokinázy vede k účinnému odstranění značek z rekombinantních fúzních LL-37 produktů obsahujících příslušnou rozpoznávací sekvenci. Kromě toho přítomnost C-terminální KDEL sekvence v kombinaci s vhodným N-koncovým signálním peptidem vedla k akumulaci produktu v proteinových tělískách odvozených od endoplazmatického retikula, které lze snadno izolovat při relativně nízkých nákladech, což činí tuto technologii ideální pro produkci antimikrobiálních peptidů pomocí rostlinného molekulárního farmaření.

Závěrem byla prokázána biologická aktivita rekombinantního LL-37 vůči *E. coli* TOP 10 buňkám, a to buď po odštěpení fúzního proteinu v případě MBP, anebo dokonce ve fúzi s menší 6xHis kotvou nebo KDEL tetrapeptidem.

Klíčová slova	antimikrobiální peptidy, biotesty, klonování, strategie genové exprese, genetická modifikace, zrnově specifické promotory, *Hordeum vulgare*, lidský beta-defensin 2, LL-37, *Nicotiana benthamiana*, pexiganan, rostlinné molekulární farmaření, detekce rekombinantních produktů
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Abstract

An alarming increase in the emergence of antibiotic resistant strains of bacteria presents a severe problem at the global scale that requires an urgent action to avoid the so called post-antibiotic era, in which bacteria may became completely resistant to treatment, thus common infections could once again kill. Antimicrobial peptides (AMPs) LL-37, beta-defensin 2, and pexiganan, represent promising control agents to treat drug-resistant infections, as they target a wide spectrum of pathogens, including those of medical importance. Despite their therapeutic potential, the use of AMPs in medicine is limited, mainly due to their high production costs. In general, the use of plants for their production seems to be beneficial in this respect. However, certain technical limitations still remain. These are basically connected to potential toxicity of AMPs to the host plant, low accumulation levels and eventually also to the issues connected to product isolation and its biological activity.

In an effort to address the above mentioned challenges, several fusion protein or peptide translocation, stabilization and purification sequences were selected and tested for their impact on accumulation level of LL-37 using transient expression in tobacco leaves. Results of this analysis provided valuable data that were taken into account when designing constructs for heterologous expression of LL-37 in barley.

Next, stable transgenic fertile barley lines expressing various codon-optimized *AMP* fusion genes either under constitutive or selected grain specific promoter were generated and analysed. Although immunolabeling using specific antibodies confirmed on protein level the accumulation of all of the designed AMPs in barley grain endosperm, it was not possible to detect recombinant pexiganan and human beta-defensin 2 using Western blot. Contrary to that, heterologous expression of human LL-37 in barley grains yielded up to 0.55 μg of recombinant peptide per gram of grain based on Western blot results. It was also shown that larger yields are achieved using a grain-specific than a constitutive promoter, that fusion of LL-37 to maltose-binding protein (MBP) increases its stability in desiccated grain and that cleavage of the LL-37 fusion protein using enterokinase results in efficient removal of the tags from recombinant products containing appropriate recognition sequence. Furthermore, the C- terminal KDEL extension in combination with N-terminal signal peptide resulted in accumulation of the product in endoplasmic-reticulum derived protein bodies that can be easily isolated for relatively low cost, which make this technology ideal for plant molecular farming with antimicrobial peptides.

Finally, the recombinant LL-37 exhibited biological activity against *E. coli* TOP 10 cells either after cleavage of the tag in the case of MBP or even in a fusion with a smaller 6xHis tag or KDEL tetrapeptide.

Keywords	antimicrobial peptides, bioassays, cloning, gene expression strategy, genetic modification, grain specific promoters, *Hordeum vulgare*, human beta-defensin 2, LL-37, *Nicotiana benthamiana*, pexiganan, plant molecular farming, recombinant product detection
Number of pages	185
Number of appendices	0
Language	English

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Objectives

1. A literature review on the topic of plant molecular farming with special emphasis on barley grain as a biotechnological tool, and also description of antimicrobial peptides and issues connected to their *in planta* expression.

2. Selection of antimicrobial peptides for recombinant production in barley.

3. Evaluation of effect of various fusion tags and localization signals on peptide accumulation.

4. Selection of promoters for gene transfer into barley.

5. Generation of transgenic barley lines expressing recombinant antimicrobial peptides and their molecular characterization.

6. Immunodetection of recombinant antimicrobial peptides in barley, their isolation and testing of antimicrobial activity.

1 Current state of knowledge

1.1 Antimicrobial peptides

Antimicrobial peptides (AMPs) are considered crucial effector molecules of the non-specific innate humoral immune response, as they act together with antimicrobial metabolites and stress related proteins during early stages of response to pathogens (Bent and Mackey, 2007). These low molecular mass peptides are evolutionary highly conserved and virtually ubiquitous in nature. Various types of peptides with antimicrobial activity have been identified across all living organisms, ranging from the simplest bacteria to human (Berry *et al.*, 2012; Harder *et al.*, 1997; Kaiserer *et al.*, 2003; Marcus *et al.*, 1997; Phelan *et al.*, 2013; Zasloff , 1987). Majority of AMPs are encoded by genes, while others are products of secondary metabolism or synthesized by non-ribosomal peptide synthases (Giessen and Marahiel, 2012). Certain AMPs are generated after post-translational modifications, have cyclic structure or contain unusual amino acids (Laverty *et al.*, 2011). Although variable in length, most of the AMPs are generally less than 50 amino acids long and can adopt a similar characteristic of forming an amphipathic structure. Traditionally, AMPs are classified into four main classes based on secondary structures that might adopt upon proper conditions. These include α-helices, β-sheets, disordered loops and extended structures (Doležílková *et al.*, 2011; Nguyen *et al.*, 2011).

Most of the AMPs possess cationic character as a result of a high number of the basic amino acid residues arginine and lysine that comprise a primary structure of AMPs. Cationic AMPs are able to selectively recognize the prokaryotic cell membranes from the eukaryotic ones due to differences in their composition. In fact, bacterial cell membranes are strongly electronegative under physiological conditions due to the presence of negatively charged phospholipid molecules, i.e. cardiolipin, phosphatidylglycerol and phosphatidylserine. Opposite to that, the overall charge of animal membranes is neutral as a result of high content of neutrally charged molecules of phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and cholesterol, respectively. Therefore, the initial interaction between cationic AMPs and prokaryotic cell membranes is based on mutual electrostatic attraction that is typically followed by pore formation, which leads to one or more of many processes including micellization, membrane depolarization,

cytoplasmic leakage, internalization of biocidal peptides or damage to intracellular macromolecule synthesis (Brogden *et al.*, 2005; Wimley *et al.*, 2010). As a result, the target cell death occurs within few minutes. This predominantly physical and unique mode of action of AMPs is associated to a very low risk of the emergence of resistant bacterial strains compared to classical antibiotics. In addition to their various degree of antibacterial properties, some of the peptides may also possess antifungal, antiviral, antiprotozoal, antitumor, insecticidal, spermicidal, chemotactic, antioxidant, anti-toxin, protease-inhibitory and ion-chanel inhibitory activities (Antimicrobial Peptide Database, http://aps.unmc.edu/AP/main.php, downloaded 22.5.2019). Together with the fact that AMPs have been shown to display low cytotoxicity to mammalian cells, there is no wonder that these small peptides, also called peptide antibiotics, present a new generation of biocidal agents for various disease treatment in human and animals, especially in an era of increasing drug resistance in bacteria caused by extensive antibiotic use. Furthermore, AMP might provide resistance to various plant pathogens including fungi, bacterial and viruses (Rahnamaeian *et al.*, 2009). Hence, they also present innovative approaches for plant protection in agriculture.

So far, more than 4 200 naturally occurring antimicrobial peptides from approximately 2 000 of various organisms have been identified (dbAMP**,** http://140.138.77.240/~dbamp/index.php, downloaded 22.5.2019), and thousands of derivatives and analogues have been computationally designed, engineered or synthetically generated using natural AMPs as templates (Laverty *et al.*, 2011). Web-based resources and databases (Table 1) provide valuable information on both natural and synthetic AMPs. These resources and databases offer convenient search tools for peptides with desired properties, provide various analysis and prediction tools, and help boost the process of discovery and design of novel AMPs with improved therapeutic index or antimicrobial properties. Moreover, certain databases might also be used for determination of a proper expression strategy for heterologous production of a target AMP (Table 1). Overall, various databases and resources not only provide information but also aid research on AMPs and allow better exploitation of biological activities of peptides in pharmaceutical as well as agricultural approaches.

Table 1: Selected list of web-based databases and resources on antimicrobial peptides (AMPs).

Database	Web site	Information and tools available	References
AMPer	http://marray.cmdr.ubc.ca/cgi-bin/amp.pl	Gene-encoded AMPs, hidden Markov models for recognition of AMP classes, novel AMP discovery	Fjell *et al.*, 2007, 2011
AntiBP2	http://www.imtech.res.in/raghava/antibp2	Server for antibacterial peptide prediction in a protein sequence	Lata *et al.*, 2010
APD	http://aps.unmc.edu/ap	Database of AMPs from various organisms, AMP prediction, novel peptide design	Wang *et al.*, 2009
BACTIBASE	http://bactibase.pfba-lab-tun.org	Natural bacteriocin database, tools for structural prediction and characterization	Hammami *et al.*, 2010
BAGEL	http://bagel.molgenrug.nl	AMPs from prokaryotes, bacteriocin mining tool for identification of candidate AMP genes	van Heel *et al.*, 2013
CAMP	http://www.camp.bicnirrh.res.in	Experimentally validated and predicted AMPs, analysis tools on sequence and structure of AMPs	Thomas *et al.*, 2010; Waghu *et al.*, 2014
DAMPD	http://apps.sanbi.ac.za/dampd	Manually curated database of AMPs, various analysis tools	Seshadri Sundararajan *et al.*, 2012
Defensins knowledgebase	http://defensins.bii.a-star.edu.sg	A database of defensin family of AMPs	Seebah *et al.*, 2007
LAMP	http://biotechlab.fudan.edu.cn/database/lamp	A database linking AMPs, various search and analysis tools	Zhao *et al.*, 2013
Norine	http://bioinfo.lifl.fr/norine	Database of non-ribosomally synthesized bioactive peptides	Caboche *et al.*, 2008
PepBank	http://pepbank.mgh.harvard.edu	Database of peptides that are 20 amino acids-long or shorter	Shtatland *et al.*, 2007
PhytAMP	http://phytamp.pfba-lab-tun.org	Database of plant AMPs, information on taxonomic, microbiological and physicochemical data	Hammami *et al.*, 2009
RAPD	http://faculty.ist.unomaha.edu/chen/rapd	Database of recombinantly-produced AMPs and expression strategies	Li and Chen, 2008

1.1.1 Human antimicrobial peptides

There are three major groups of host defense AMPs which can be found in human body, all of them playing a critical role in warding off invading microbial pathogens (Wang and Guangshun, 2014). These include cathelicidins, defensins and histidine-rich cationic linear histatins that can be found in human saliva (MacKay *et al.*, 1984), where they protect human body against oral infections. The group of cathelicidins and defensins will be described in more detail below, as genes coding for these recombinant AMPs were used for plant transformations in scope of this thesis.

Cathelicidins are members of mammalian AMPs, which are deposited in neutrophil granulocytes and macrophage cells, from where they are released upon leukocyte activation. In addition to the cells of the myeloid series, the presence of cathelicidins has also been described, for example, in the epithelial cell, where they are not stored, but continuously expressed and immediately released. Catelicidins are synthesized in the form of inactive prepropeptides consisting of: 1) the variable N-terminal signal sequence responsible for subcellular localization, 2) relatively conserved cathelin domain with considerable interspecies homology, 3) and C-terminal antimicrobial domain with high intra- and inter-species variability (Kościuczuk *et al.*, 2012). In humans, the *CAMP* gene (Cathelicidine Anti-Microbial Peptide) represents the only one gene coding for the cathelicidin protein member that has been described so far. The *CAMP* gene is located on chromosome 3, is approximately 2 kb in size and contains 4 exons. The gene product is known as the antimicrobial protein hCAP-18 that represents the precursor molecule from which the C-terminal antimicrobial domain known as LL-37 antimicrobial peptide can be released by proteinase 3 cleavage (Gudmundsson *et al.*, 1996). LL-37 is a small cationic 4.5 kDa peptide of 37 amino acids, overall charge +6, and an isoelectric point of 10.6. 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 (Kościuczuk *et al.*, 2012). LL-37 mediates a variety of biological functions. Its activation reflects the body's response to infectious and inflammatory stimuli or various injuries (Hancock and Diamond, 2000). The antimicrobial spectrum of the peptide covers a variety of both G+ and G- bacteria including pathogens of medical importance (e.g. multidrug resistant strain of *Pseudomonas aeruginosa* or methicillin-resistant *Staphylococcus aureus*)

(Turner *et al.*, 1998). Minimum inhibitory concentrations (MIC) of the peptide are often in the submicromolar range (Larrick *et al.*, 1995), wherein the peptide is particularly active against G-bacteria, due its high affinity for negatively charged lipopolysacharides (LPS) presented in the outer layer of their cell wall. The alpha helix represents active structural motif of LL-37 (Fig. 1). Before the peptide reaches bacterial cell, it does not possess any defined structure and LL-37 became helical based on the pH value (with highest increase at neutral pH), LL-37 peptide's concentration, ionic strength (Johansson *et al.*, 1998) and upon its interaction with biological membranes. Nuclear magnetic resonance (NMR) spectroscopy studies showed that residues 1-31 of LL-37 are necessary for the peptide binding properties to LPS, which is not true for its C- terminal tail (Wang, 2008). The presence of hydrophilic S9 in the structure of LL-37 is responsible for separation of hydrophobic surface of the alpha helix into two domains, namely the N-terminal short helix and the long central helix. As a result, the LL-37 peptide exhibits a cooperative binding dynamics to bacterial LPS (Lehrer *et al.*, 1998). The central helix is considered as the key antimicrobial region responsible for biological properties of LL-37 (Fig. 1). In addition to antibacterial properties of LL-37 including inhibits of bacterial biofilm formation, the peptide also prevents infections of both viral (Currie *et al.*, 2013) and fungal (Ordonez *et al.*, 2014) origin, inhibits tumorigenesis (Okumura *et al.*, 2004), induces mast cell chemotaxis (Niyonsaba *et al.*, 2002) cellular apoptosis (Barlow *et al.*, 2006), or possess wound healing properties by stimulating epithelial cells angiogenesis and recovery (Carretero *et al.*, 2008). Regarding plant systems, the gene coding for cathelicidin LL-37 or its variant was integrated into genome of Chinese cabbage (*Brassica rapa* var. *chinensis;* Jung *et al.*, 2012), tomato (*Solanum lycopersicum;* Jung, 2013), and rice (*Oryza sativa* L. var. Japonica cv. Dongjinbyeo; Lee *et al.*, 2017) 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.

Defensins are evolutionary ancient, arginine-rich, small (4-6 kDa) cationic peptides found in various living organisms including humans and other mammals, fishes, birds, insects, fungi and plants (Liu *et al.*, 1997). Based on the composition of their primary structures and disulfide bridges formation are the human defensins divided into two distinct groups, namely the alpha-defensins and the beta-defensins, respectively. In the subgroup of alpha-defensins, disulfide bridges are typically formed between cysteines 1-6 / 2-4 / 3-5, while in the beta-defensin subgroup they are between cysteines 1-5 / 2-4 / 3-6 (Winter and Wenghoefer, 2012). So far, 6 of the human alpha-defensin peptides have been described. They are also known as human neutrophil peptides (hNP1-hNP6), as they are predominantly found in neutrophil granules, where they are constitutively expressed. Besides, the alpha-defensins are also expressed in the cells of the small intestine (the so-called Panet cells), natural killer cells, monocytes, and some of the T cells (Ganz, 2003). In addition, their presence has also been detected in respiratory epithelium and in a female urogenital tract (Quayle et al., 1998). Regarding the group of the human beta-defensins, more than 26 genes coding for them have been predicted based on human genome sequencing results. However, only few of the gene products have been characterized on protein level (Schutte *et al.*, 2002). They are basically expressed in epithelial cells of urogenital, gastrointestinal and respiratory tract (Klotman a Chang, 2006), as well as in the cells of epididymis (Yamaguchi *et al.*, 2002). All of the defensins are synthesized as prepropeptides consisting of approximately 110 amino acids. The precursors contain a conserved N-terminal signal sequence of 19 amino acids plus a C-terminal antimicrobial domain of 29 to 34 amino acid residues (Daher *et al.*, 1988). Such minimum chain length is essential to maintain their biological activity. During neutrophil differentiation, hNP1-4 prepropeptides are digested by elastase and proteinase 3 (Tongaonkar *et al.*, 2012). The resulting mature alpha-defensins are subsequently stored in azurophilic granulocytes, where they represent about 40% of total protein content (Gabay a Almeida, 1993). In case of pathogen attack, hNPs are released into phagocytic vacuoles, where they represent one of the most essential components for fighting infections. Interestingly, the precursor molecules for hNP5 and 6, respectively, could be digested by various proteases into mature peptides of variable length, which are presented in female reproductive system (Quayle *et al.*, 1998). Defensins are characterized by relatively compact structure composed of a rigid beta sheet that is stabilized by 3 disulfide bridges (Figure 2; Selsted *et al.*, 2005).

However, the group by Wu *et al.* (2003) demonstrated that both the alpha and the beta defensins, when linearized, maintain their biological activity, hence the presence of disulphide bonds may not be always crucial for retaining of the antimicrobial properties. Defensins exhibit activities against range of pathogenic bacteria including *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Streptococcus faecalis, Candida albicans* and others (Ganz *et al.*, 1985; Wilde *et al.*, 1989). In addition to their antibacterial properties, some of the defensins also exhibit antiviral activities either via impacting of viral entry, or by various mechanisms that negatively affect virus post its entry. Relatively well has been described the effect of defensins on human immunodeficiency virus (HIV), that is based on interaction of these AMPs with membrane / envelope glycoproteins (Wang *et al.*, 2004). Some of the defensins might exhibit their antiviral activity by direct interaction with cell surface receptors of the infected cell. As a result, they block the intracellular signaling cascade responsible for replication or transcription of viral particles. In addition, the antiviral effect of defensins might be mediated via inhibition of releasement of virions from endocytic vesicles; via causing virions to agregate and thus to impeding cell binding or to prevent cell entry; via inhibition of fusion of viral lipid bilayer with that of the infected cell or through inhibition of penetration of the host cell, and many others (Wilson *et al.*, 2013). Defensins have also been shown to exhibit wound healing properties. For example, the expression of the human beta-defensin 2 (HBD2), also known as skin-antimicrobial peptide 1 (Figure 2) is elevated at the site of skin injury and in chronic wounds, where they promote the expression of anti-inflammatory cytokines (Butmarc *et al.*, 2004; Roupe *et al.*, 2010). The urrogenital tract, in particular the male reproductive system, represent the major sites of the expression of most of the beta-defensins, whose levels varies depending on age, with the highest peak by the period of sexual maturity (Patil *et al.*, 2005). They are able to bind to the sperm plasma membrane and therefore to protect sperm cells against various pathogens in both the male and the female genital tract (Zhou *et al.*, 2004). Since the human beta-defensin 2 represent a promissing drug candidate, many researchers aimed its recombinant low-cost large scale production using various strategies in different host organisms including bacteria (Rao *et al.*, 2004; 2005), yeasts (Chen *et al.*, 2011) and plants (Aerts *et al.*, 2007).

A

LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES

B

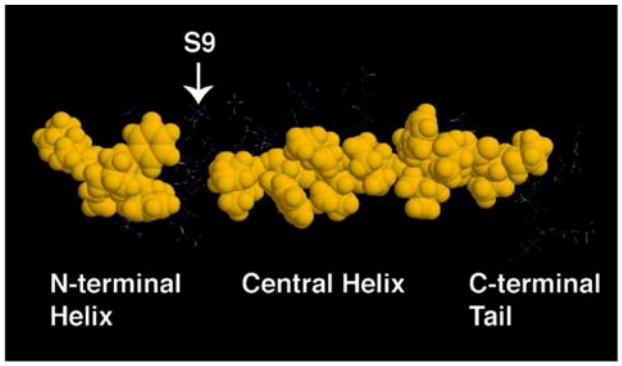

Figure 1. Structural properties of LL-37. Primary sequence of LL-37 with the central arginine R23 (indicated in red) being as a part of long central helix of particular importance in binding to bacterial membranes and DNA (A). NMR 3D structure of LL-37 showing a separation of the long amphipathic helix by a hydrophilic residue serine 9 into the N- terminal short helix and the long central helix as the major antimicrobial region (B) (Wang *et al.*, 2014).

A

GIGDPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP

B

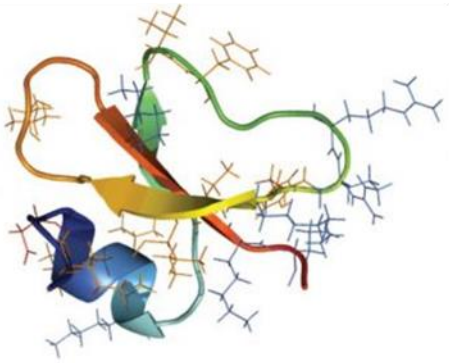

Figure 2. Structural properties of human beta-defensin 2 (HBD2). Primary sequence of HBD2 (A). Rigid, triple-stranded, antiparallel beta sheet structire of HBD2 with an alpha helix at the N- terminus of the peptide (B) (smart-bioscience, 2019).

1.1.2 Antimicrobial peptides of *Xenopus laevis*

In model organism *Xenopus laevis*, two types of peptides with antimicrobial activity have been isolated and described so far, namely the magainins and the alpha-helical PGQ peptides, which are expressed in the stomach of the African clawed frog and stored as active, processed peptides in a novel granular multinucleated cell in the gastric mucosa (Moore *et al.*, 1991). Next section will be focused on more detailed description of magainins, as gene coding for their analogue, namely the pexiganan, was heterologously expressed in barley in scope of this thesis.

Magainins represent a family of linear, cationic antimicrobial peptides that are composed of 21 to 27 amino acid residues. They are found in the skin of African clawed frog, where they were accidentally discovered in frame of study of *Xenopus laevis* oocytes genes expression, which were collected from anesthetized adult females from the abdominal cavity. The incisions were made through the skin into the lower abdominal. Interestingly, although the whole procedure was performed under non-sterile conditions, there have very low number of frogs developed infection diseases at the site of incision after their returning back to contaminated water, which reflected the presence of compounds with antimicrobial properties (Zasloff *et al.*, 1987). So far, two types of magainins have been identified, magainin 1 and magainin 2, respectively, which slightly differ in their amino acid sequence composition. Magainin 2 represnet 23 amino acid residues long (Figure 3) toroidal pore forming antimicrobial peptide (Imura *et al.*, 2008), that was found to act as a broad spectrum antimicrobial agents. Some of the examples of its potential practical use include the production of spermicidal agents with dual activities, namely the spermicidal plus the microbicidal. This combination might bring the solution to reduce the ever growing number of people infected with HIV. In addition to their activity against HIV, magainins might help the fight in combating other sexually transmitted diseases caused by flagellates, chlamydia or the Herpes simplex virus (Zairi *et al.*, 2009). Moreover, magainin 2 might be used for the treatment of diabetics due to its insulin-releasing properties leading to reduction of free glucose level in blood stream (Ojo *et al.*, 2014) or eventually also for the treatment of cancer patients due to its tumoricidal properties (Anghel *et al.*, 2013). Due to high therapeutic potential of magainin 2, many of its synthetic analogues have been designed with the aim

to maximize its broad spectrum activity and to develop clinically useful therapeutic compound. Zasloff *et al.* (1988) have shown that removal of amino acid residues from both N- as well as C- terminus of magainin 2 negatively affected its antimicrobial properties, meaning that some minimal peptide length was essential for retaining of its properties. It was also found that the peptide's helicity correlates with its activity and that Gly to Ala substitution increased the α-helical content and as a result also improved the peptide antimicrobial properties (Chen et al., 1988). In addition, amidation on C- terminus plus removal of Glu19 was also shown to increase peptide activity (Cuervo *et al.*, 1988). These information resulted available resulted in the development of Pexiganan (also known as MSI-78), which represents a 22 amino acid long analogue of the antimicrobial peptide magainin 2 (Figure 3). A lot of efforts have been made to develop proper strategy for recombinant low-cost production of magainins and their derivatives. For example, C- terminal fusion of magainin 2 to the family III carbohydrate-binding module, to whose N- terminus the linker sequence from *Clostridium thermocellum* has been linked, led to successful production of magainin 2 in *E. coli* cells. In addition, a formic acid recognition site was introduced between the two modules, which allowed chemical cleavage of the peptide followed by its release in its bioactive form (Ramos *et al.*, 2013). Next, a study by Zhao and co-workers (2015) describes a low-cost chromatography-free technology for production of pexiganan in *E. coli* that was based on fusion of pexiganan to the DAMP4 biosurfactant protein, via an acid-sensitive cleavage site. As a result, simple thermal cell-breakage and purification based on precipitation followed by acid cleavage was sufficient for recovery of purified biologically active pexiganan. Another study by Jang and co-workers (2009) reports production of pexiganan in *E. coli*, that was translationally coupled to the anionic derivative of human gamma interferon that efficiently neutralized the AMPs toxicity. Concerning plant systems, combination of the modified AMPs based on indolicidin and magainin have been expressed in *Nicotiana tabacum* and *Arabidopsis thaliana* plants. As a result, transgenic plants showed enhanced resistance to various plant pathogenes (Xing *et al.*, 2006).

A

Magainin 1: GIGKFLHSAGKFGKAFVGEIMKS
Magainin 2: GIGKFLHSAKKFGKAFVGEIMNS
Pexiganan: GIGKFLKKAKKFGKAFVKILKK - NH_2

B

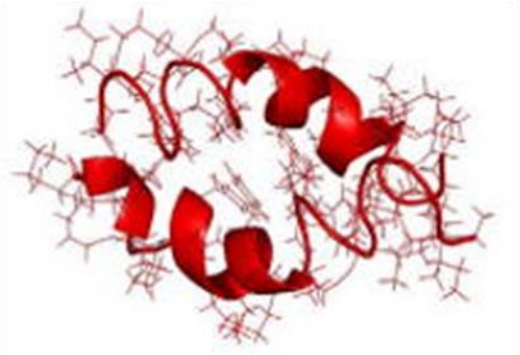

Figure 3. Structural properties of magainins and their derivative pexiganan. Primary sequences of magainin 1, 2 and pexiganan (A). Antiparallel dimeric helical structure of pexiganan that is created in a membrane environment and represents a key step in its activity (B) (Gottler and Ramamoorthy, 2009).

1.2 Plant molecular farming

Modern medicine continuously faces incidences of resistance among pathogenic microorganisms to commonly employed antibiotics, thus it encourages the search for novel strategies and new antimicrobial agents for treatment of pathogenic diseases. Various AMPs are making their way as potential novel therapeutics for both human and animals (da Rocha Pitta *et al.*, 2010; Peters *et al.*, 2010). Commercial large-scale production of AMPs with high purity is required for practical approaches in microbial disease treatment. However, the lack of large-scale cost-effective production technology of AMPs represents one of the main barriers for their everyday routine use in medical practice. One of the promising strategies includes the so-called plant molecular farming (PMF), where plant cells or tissues are used for expression and production of recombinant pharmaceutical proteins or peptides. Plants address advantages of mammalian or microbial cell culture methods but lack their pitfalls (Basaran and Rodriguez-Cerezo, 2008). First and foremost is the possibility of commercial production of high-value compounds within the growing plant tissues for lower costs compared to other heterologous expression systems (Fischer *et al.*, 2004; Ma *et al.*, 2003). This is a consequence of the fact that plants gain energy from sunlight,

and use raw materials or minerals from soil, water, and air. Plants as production platforms are considered safe regarding biosafety, since there is a low to no risk of product contamination by animal or human viruses and pathogens. This is due the fact that plants generally cannot harbor these pathogens (Daniell *et al.*, 2001; Fischer *et al.*, 2004). Additionally, use of plants for commercial production avoids the ethical case deliberation of animal expression systems. Following the benefits mentioned above, plants are able to perform complex eukaryotic posttranslational modifications like glycosylation or *SS*-bond formation required for production of functional proteins structurally similar to their native counterparts (Ma *et al.*, 2003; Ramessar *et al.*, 2008).

Genetically modified (GM) plants have provided tremendous advances in crop improvement and molecular farming. Various crop plants including maize, alfalfa, soybean, rice and others have been engineered and commercialized over the last few decades (ISAAA, 2019). Heterologous expression of proteins and peptides in GM plants are mainly performed for improved agricultural practices, cost-efficient crop production and/or increased crop yield and quality. Traits engineered in most of the GM plants include herbicide, insect or disease resistance. On the other hand, progress in biotechnology has enabled plant-based expression of various biological macromolecules and polymers bearing critical importance in medicine, pharmacy and industry. These applications have introduced new areas of utilization for plants besides their conventional uses as food and feed. Various effective plant-based expression platforms range from plant cell suspensions to field-grown transgenic plants (Twyman *et al.*, 2003). Tobacco and potato were used as model plants for production of antigens, proteins and pharmaceuticals in earlier studies (Arakawa *et al.*, 1997; Sijmons *et al.*, 1990; Thanavala *et al.*, 1995). 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.

Although new generation of GM plants moves forward via employment in PMF, heterologous expression of AMPs in plants for molecular farming has been limited. Technical restrictions leading to low product yield and instability still await innovative solutions (Obembe *et al.*, 2011). Furthermore, plant-based AMP production faces limitations in authorization processes by local legislatives and concerns raised

by public. Field trials of novel GM plant varieties are often smashed by activists opposed to GM crops in Europe (Kuntz, 2012). The doubts of opponents are linked not only to ethical deliberations, but also to a risk that modified genetic material might accidentally end up in the food chain due to cross-pollination or horizontal gene transfer (McHughen and Wager, 2010). Additionally, food crops such as maize employed for production of pharmaceuticals might simply mix with counterparts and contaminate food supply, as in the case of ProdiGene incident (Fox, 2003).

Field-grown plants on agricultural scale are advantageous in terms of production capacity and cost. However, pharmaceutical companies using plant biotechnology are struggling with strict regulations on field-grown GM plants. Utilization of self-pollinating plants and geographic separation of these transgenic crops in tightly isolated fields away from other agricultural practices might provide feasible solutions to comply with regulations. Although a wide range of platforms for AMP molecular farming in plants is available, considerable effort needs to be invested in choice and improvement of optimal production strategy to fulfill the requirements for economic feasibility and comply with the legislative regulations for handling GM plant material.

Athough not connected to transgene insertion, it is worth to mention on this place the new generation of crop plants obtained by modern methods of precision gene-editing techniques (also called new breeding technologies) such as CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/associated protein 9), that have been subject to intense discussions over recent years. In July 2018, depsite their high potential, the European Court of Justice (ECJ) ruled that gene-edited crops are genetically modified organisms which means that they are subject to the same stringent regulations as conventional GM crops. However, scientific communities from more than 120 European institutions incuding the Czech researches from the Centre of the region Haná for Biotechnological and Agricultural Research (CRH) appeal to the European Union for a change of legislation on these crops, as they find this directive as a step back that will limited planting and sale of these crops, hence significantly slow down European research in this area and move this focus of research somewhere outside Europe. They argue that plants created via targeted genome editing techniques are as safe as plants derived from conventional breeding techniques such as mutagenesis caused by e.g. irradiation. These plants already exempt

from the law, as they do not contain artificial DNA sequences or DNA from other species, which is also truth for the genetically edited crop plants. In addition, scientists believe that this progressive technology represents a revolution in the field of molecular biology that could adress the issues of global warming, food safety as well as sustainability, e.g. via creation of plants with increased yields or plants being more resistant to drough stress or pathogen attack. Hence, it is highly desirable to change the directive behind the gene-edited crops to gain the opportunity to meet these needs (Callaway, 2018; Hundleby and Harwood, 2019; Polčáková, 2019).

1.2.1 Barley grain as a tool in plant molecular farming

Since seeds of diverse plants are consumed by human as well as animals, accumulation of AMPs in these storage organs represents a promising approach for development of plant-derived edible biopharmaceuticals. Moreover, the use of barley, especially its grain, as a tool for molecular farming has emerged as a favorable strategy, as it is a plant storage organ whose natural properties enable efficient, robust, and scalable heterologous production of desired compounds. Furthermore, barley grains provide biochemically inert and stable environment which allows long term storage of recombinant proteins or peptides without loss of product quality, and enable simplified downstream processing (Stöger *et al.*, 2002; Xu *et al.*, 2012). From the chemical point of view, this end-use organ is comprised of four major constituents namely carbohydrates, proteins, minerals, and phytochemicals. Carbohydrates represent about 80% of the mature barley grain (MacGregor and Fincher, 1993). Starch, the product of photosynthetic carbon fixation, comprises about 50-70% of barley grain (Henry, 1988). It is presented in the form of starch granules mainly in the endosperm where it is the major compound. Starch can be accumulated also in other parts of barley grain such as embryo, aleurone, or pericarp; however its presence in these organs occurs only transiently during various stages of grain development (Radchuk *et al.*, 2009). Proteins are the second most abundant group of barley seed components. Their presence is within the range of 8-15% on a dry-weight basis, and they are mainly accumulated in endosperm. Barley seed proteins can be classified according their functions as structural, storage, or metabolic and protective proteins. Another possible classification is based on extraction properties of proteins, an approach established by Osborne (1895). According this classification,

there are four different protein fractions in barley grains, namely albumins (4% of total soluble protein, TSP), globulins (18% of TSP), prolamins (37% of TSP), and glutelins (37% of TSP) (Gubatz and Shewry, 2010). An overview of dominant polypeptide classes is presented in Fig. 4. For the purposes of PMF, knowledge on types of barley seed proteins is essential, as their promoters might be useful tools for driving strong, seed-preferable expression of transgenes. Barley grains present various tissues suitable for expression and deposition of heterologous products. The most widely used target tissue is endosperm followed by aleurone layer. So far, certain promoters of aleurone- or endosperm-specific genes have already been proved to be versatile vehicles able to direct expression of antigens, human proteins and growth factors, technical enzymes or other recombinant proteins into these distinct barley tissues (Erlendsson *et al.*, 2010; Horvath *et al.*, 2000; Joensuu *et al.*, 2006; Stahl *et al.*, 2002).

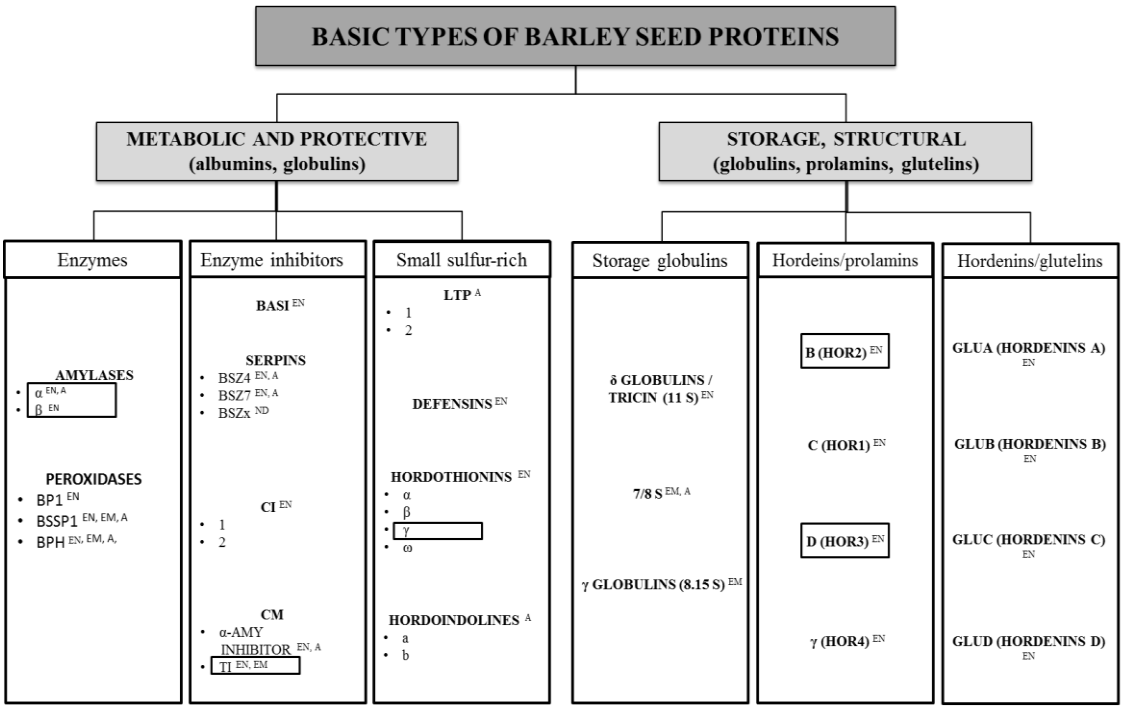

Figure 4. Schematic summary of the basic groups of mature barley seed proteins.

Classification is based on protein function. Proteins whose gene promoters have already been used for the purpose of molecular farming in barley grains are marked in boxes. A, aleurone; α-AMY, α-amylase; BASI, barley α-amylase/subtilisin inhibitor; BP1, barley peroxidase 1; BPH, barley peroxidase homolog; BSSP, barley seed specific peroxidase 1; CI, chymotrypsin inhibitor; CM, chloroform methanol extracted proteins; EM, embryo; EN, starchy endosperm; LTP, lipid transfer proteins; ND, non-determined, SERPINS, serin protease inhibitors; TI, trypsin inhibitor. Adapted from Eggert *et al.*, 2010; Gubatz and Shewry, 2010; Hayes *et al.*, 2003; Roberts *et al.*, 2003.

Similar to other cereal seeds, developing barley grains possess efficient protein machinery with a rich mixture of different enzymes that enable correct folding of the heterologous protein. Moreover, a key advantage of seed cellular background is the presence of various types of protease inhibitors. The primary biological role of protease inhibitors is proposed to be connected with defense, as these inhibitors are able to block exogenous proteases from different plant pathogens that are responsible for breakdown of barley seed proteins (Jones and Fontanini, 2003; Pekkarinen *et al.*, 2007; Roberts *et al.*, 2003). Some of the protease inhibitors might also help the seeds to pass unharmed through the digestive tract of animals that feed on whole plants, thus help the plant to be distributed (Mikola and Suolinna, 1969). Some of the examples for protease inhibitors include Bowman-Birk type trypsin inhibitor (BBBI), α-amylase/subtilisin inhibitor (BASI), chymotrypsin inhibitor 2 (CI-2), and different types of serpins, as well as the proteins extracted by chloroform and methanol, so called CM proteins. Besides their functions in defense, some of these may protect barley grain cells against endogenous proteases, and thus help the seed to avoid autolysis throughout dormancy stage (Jones and Fontanini, 2003; Pekkarinen *et al.*, 2007; Roberts *et al.*, 2003). Low protease content in barley grain together with a low content of water during dormancy allow long-term storage of heterologous proteins of interest at ambient temperature without loss of activity (Eskelin *et al.*, 2009; Patel *et al.*, 2000). Therefore, it is possible to decouple the process of protein production from the process of subsequent downstream processing. Extraction and purification of the heterologous products are largely assisted by the fact that barley grain has relatively low content of secondary metabolites, is free of endotoxins, and has a simple protein profile. Most of the barley proteins appertain to few main polypeptide fractions (Fig. 4). Thus, highly efficient recovery of recombinant proteins may be achieved by simple separation techniques commercially available.

Moreover, barley holds certain agronomical advantages. There are powerful methods available for harvest, transport and storage of barley grains. Last but not least, domesticated diploid barley is a self-pollinating species. Thus, outcrossing with other non-transgenic plants is extremely rare (Ritala *et al.*, 2002). Additionally, barley holds the GRAS (generally regarded as safe) status from the U.S. Food and Drug Administration (FDA). Furthermore, after homozygous transgene fixation, it is also

possible to grow the seeds on a field, and thus, increase the amount of recombinant product logarithmically. Taken together, barley grains provide unique features that address many of the drawbacks of previously established other expression systems. Choice of barley grains as a biomanufacturing platform can be a valuable strategy for low-cost and large scale production of recombinant proteins and pharmaceuticals.

1.2.1.1 Promoters for grain specific expression in barley

For the purpose of PMF, achievement of high levels of recombinant products in desired plant tissues is crucial. Although transgene expression and target production can be increased by optimization of various parameters, choose of optimal promoter suitable for molecular farming hold the key to match the requirements for high protein accumulation. The use of promoters able to drive tissue-specific expression possesses several benefits over exploiting their ubiquitous counterparts. Proteins recombinantly produced in all parts of plant body may have negative pleiotropic effects on the vegetative growth, and thus influence yield (Hood *et al.,* 2003). With the use of strong grain-specific promoters, it is possible to achieve higher accumulation levels of proteins in seeds compared to ubiquitous promoters. For example, maize constitutive ubiquitin-1 (*ZmUBI-1*) promoter was compared with rice endosperm-specific glutelin B-1 *(OsGLUB-1)* promoter in regard to product accumulation in barley T1 grains. Average accumulation amounts of recombinant products under the control of *ZmUBI-1* promoter were 3- to 50-fold lower than that under the control of *OsGLUB-1* promoter (Eskelin *et al.,* 2009).

Since the barley endosperm is much larger than other tissues, and is also the major site for protein deposition, the largest group of promoters widely used in PMF are endosperm-specific. Most of the endosperm-specific promoters are derived from seed storage protein genes of barley or other cereals. One of the most commonly used endosperm-specific promoters is rice *OsGLUB-1* promoter (Eskelin *et al.*, 2009; Kamenarova *et al.*, 2007; Patel *et al.*, 2000). Using this promoter, it has been shown that it is possible to produce different industrially important compounds. For example, Patel *et al.* (2000) have demonstrated the possibility to generate and stably store the cell wall degrading enzyme xylanase in developing barley grains. Xylanase improves the efficiency of feed grain conversion in monogastric animals. Expression of the fungal

chimeric xylanase gene was driven under the control of *OsGLUB-1* or barley hordein B-1 (*HOR2-4*) promoters. Additionally, transformation vectors also contained rice *rbcS* 3' region (region of the small subunit of the rice ribulose-1,5-bisphosphate carboxylase) adjacent to the right border of T-DNA. Although hordeins in ripe grain form about 50% of the total endosperm protein, the activity of recombinant xylanase was at least 2-fold higher in *OsGLUB-1* than that in *HOR2-4* transgenic lines. The rice *GLUB-1* promoter has also been used in another comparative study on ER-targeted expression of full-length gene coding for collagen α-1 chain (*COL1A1*) and its more stable 45-kDa fragment in barley seeds. Both genes were optimized for monocot expression. In one of the homozygous doubled haploid lines, accumulation levels of 45-kDa COL1A1 fragment reached 0.07% of total extractable protein. Whereas, accumulation of this collagen fragment driven by barley germination-specific aleurone α-amylase (*α-AMY*) promoter reached only 0.028% of total extractable protein in T0 lines. Some of the expression cassettes also contained so called epsilon-element from the Cocksfoot mottle virus for evaluation of its function as a translation enhancer. The element was used as a 5' leader sequence and placed between N-terminal signal sequences and the promoter region. However, authors evaluated impact of the element on enhancing the expression as negligible (Eskelin *et al.*, 2009). In other studies, *OsGLUB-1* promoter was successfully used for production of human lactoferrin in barley grains (Kamenarova *et al.*, 2007; Tanasienko *et al.*, 2011).

Another strong endosperm-specific promoter suitable for PMF is barley endogenous hordein D (*HOR3-1*) promoter (Sörensen *et al.*, 1996). The possibility to employ *HOR3-1* promoter to direct endosperm-specific expression of the gene coding for structural E2 protein of the CSFV (classical swine fever virus) was patented by Nelsen-Salz *et al.* (2003). The immunogenic E2 protein serves as a vaccine against the mammalian CSFV. According to their invention, the codon of the gene for E2 was subjected to optimization for the barley host plant, and the gene was fused with hordein D signal peptide, and placed under *HOR3-1* promoter. In addition, barley *HOR3-1* promoter is one of the promoters used by ORF Genetics Ltd. (Iceland) for commercial production of biologically active recombinant human proteins with a yield analogous to prokaryotic expression systems. Next, Erlendsson *et al.* (2010) published a report describing *HOR3-1* promoter-driven production of codon-optimized human FLT3 ligand that was fused to an N-terminal polyhistidine tag for affinity purification.

In the highest producing line, the estimated level of recombinant product reached up to 60 mg kg^{-1} of T1 seeds. The *HOR3-1* promoter was also used by Horvath *et al.* (2000), where expression of an engineered termostable endo-1,4-β-glucanase was achieved in barley endosperm tissues. Accumulation of heat stable product in homozygous T2 seeds reached, on average, 5.4% of the extractable proteins.

A patented report describes the use of barley *HOR3-1* promoter for production of natural sweetener thaumatin from African perennial herb *Thaumatococcus daniellii*. Authors prepared a construct, where GC-optimized thaumatin sequence was fused on the N-terminus with hordein D signal sequence. In another experiment, the same inventors used a different strategy for production of this compound. They constructed a transformation vector containing a sequence of *HOR3-1* promoter upstream of the sequences for N-terminal thaumatin signal peptide, the thaumatin itself, and C-terminal thaumatin signal peptide (Stahl *et al.*, 2009a). In another study, barley *HOR3-1* promoter was used to control the expression of a sequence coding for a hybrid protein comprised of codon-optimized human homeobox B4 protein fused with carbohydrate binding module from *Thermotogota maritima*. Inventors of this patented report described yet another strategic tool to maximize production of the chimeric protein in barley. Their approach was based on suppression of abundant barley storage proteins, hordein B and C, as there is always competition for limited amount of different resources such as amino acids and translational machinery. Promoters of the hordein B and C harbor GCN4-like motif, which is recognized by BLZ1 and BLZ2 transcription factors. Authors prepared a binary construct for posttranscriptional silencing of *BLZ1* and *BLZ2* genes, and consequently, aimed to reduce abundance of hordein B and C. Since the *HOR3-1* promoter does not contain the GCN4-like motif, its function is not influenced by the silencing (Orvar, 2005).

Successful production of an edible vaccine in barley endosperm for porcine against F4-positive enterotoxigenic *Escherichia coli* (ETEC) was reported. In this study, immunogenic fimbral adhesin (FAEG), with adhesion to F4, was produced in endosperm and shown to be heterogeneously glycosylated and immunologically active. Effects of 3 different barley endosperm-specific promoters, namely *HOR2-4*, β-amylase (*β-AMY*), and trypsin inhibitor (*TI*) were evaluated. Using GUS expression, the *TI* promoter was determined as the most active in endosperm tissue. Hence it was

employed for high level accumulation of ER-targeted recombinant FAEG. The expression of FAEG protein yielded up to 1% of grain total soluble protein (TSP). Effective production was achieved by fusion of FAEG coding sequence with an ER-targeting signal peptide (SEKDEL) of barley TI and translation enhancer elements, namely the epsilon-element from the Cocksfoot mottle virus and the 5' untranslated exon and first intron of maize ubiquitin (Joensuu *et al.*, 2006).

The barley *γ*-hordothionin promoter also belongs to promoters that have been used so far for the purpose of molecular farming in barley kernels. This promoter was employed to drive expression of codon-optimized human serum albumin gene that was produced as a fusion protein with *γ*-hordothionin N-terminal signal peptide (Stahl *et al.*, 2009b). Next, the promoter derived from a gene coding for wheat endosperm-specific high-molecular-weight glutenin Bx17 (HMW Bx17) was used to target expression of an anti-glycophorin single-chain antibody fused to an epitope of the HIV, which might be used as a reagent for detection of the virus in blood, into the barley endosperm tissue. The fusion antibody was expressed with C-terminal ER-retention motif KDEL and N-terminal legumin signal peptide, which ensured transport of the recombinant protein via the lumen of ER and its retention there. Using barley endosperm-specific expression strategy, high-level expression (150 μg of antibody g^{-1} grain) was achieved (Schünmann *et al.*, 2002). To complete the list on most promissing promoters suitable for molecular farming in barley grains, it is important to point out the oat globulin 1 *(AsGLO1)* promoter that was used by the group of Hensel and co-workers (2015) for synthesis of an anti-HIV monoclonal antibody in barley endosperm tissue. Authors of this study fused the legumin B4 signal peptide and the ER-retention signal SEKDEL to the N- and C- terminus of the recombinant antibody, respectively. Using this strategy, the best features doubled haploid transgenic lines accumulated up to 160 μg of the recombinant protein per g of barley grain, which corresponded to the 0.4 % of the total soluble proteins and the recombinant product was localized along the periphery of the protein bodies.

1.3 Antimicrobial peptide production in plants

Recently, the interest in expression of AMPs in plants has significantly increased because of two main reasons, namely the need for novel approaches in plant protection and the demand for new antimicrobial agents in medicine. Natural and synthetic AMPs are considered prospective candidates which might address these demands via various approaches in agricultural and medical biotechnology. Although promising, production of AMPs in plants has been challenging because of particular physiochemical properties of these peptides. Hence, specific expression strategies leading to higher yield and stability of AMPs are often employed together with optimization of AMP activity against target pathogen groups and alteration of toxicity to host and non-target organisms. Current progress in the field of AMP production in plants is described in more detail in following sections.

1.3.1 Antimicrobial peptide expression in plants to confer disease resistance

Crop plants are susceptible to various diseases caused mainly by diverse pathogenic fungi or bacteria. This contributes to substantial loss in yield and quality of agricultural products, which leads to significant economic loss worldwide (Abdallah *et al.*, 2010; Coca *et al.*, 2006; Rahnamaeian *et al.*, 2009; Rivero *et al.*, 2012; Zakharchenko *et al.*, 2013a; 2013b; Zhou *et al.*, 2011). Crop loss due to plant pathogens and pests reach 30 – 40% in developing countries annually (Flood, 2010). Nowadays, bacterial and fungal disease control mainly relies on agrochemicals including classical antibiotics such as streptomycin and fungicides such as copper containing compounds. These chemicals may have negative impact on human health and environment. Moreover, extensive use of agrochemicals can induce pathogen resistance which makes conventional pesticides less effective. Therefore, strategies based on development of disease resistant plant varieties expressing natural or synthetic AMPs of plant or non-plant origin (Table 2) can significantly reduce the use of chemicals in agriculture, which in turn might be appreciated by environmentalists and customers.

To develop protection against plant pathogenic bacteria and fungi, heterologous expression of natural or *de novo* designed AMPs has been successfully used in an array of diverse agronomically important crops (Table 2). Introduction of genes coding

for certain AMPs can also induce resistance to insect pathogens. Expression of *Brassica rapa defensin 1* transgene in rice resulted in strong resistance to sap-sucking insect, brown planthopper (Choi *et al.,* 2009). Furthermore, AMPs, expressed in plants and integrated in overall protective systems, not only provide defense against biotic stresses, but also might induce protection against abiotic stresses. It was demonstrated that expression of a gene coding for cecropin P1 in rapeseed increased tolerance to oxidative stress caused by herbicide paraquat (Zakharchenko *et al.*, 2013a). The same gene was used for transgenesis of camelina, where *cecropin P1* expressing plants exhibited increased sustainability under salt stress (Zakharchenko *et al.*, 2013b). Improved tolerance to both biotic and abiotic stress factors might be explained by probable function of cecropin P1 or certain other AMPs in up-regulation of expression of endogenous genes involved in general stress response (Campo *et al.*, 2008). Interestingly, accumulation of AMPs in plants might also improve plant productivity. For example, transgenic tobacco lines expressing an indolicin-derived peptide displayed increased biomass production in field trials (Xing *et al.,* 2006).

Table 2: Selected list of antimicrobial peptides expressed in plants for plant protection (PP). ND: not determined.

Peptide name	**Description and peptide origin**	**Plant host**	**Expression system and strategy**	**Promoter**	**Application**	**Yield**	**References**
AttA	Attacin A from *Tricloplusia ni*	*Citrus sinensis* (sweet orange)	Constitutive	*CaMV35S*	PP against *Xanthomonas axonopodis* pv. *citri*	ND	Boscariol *et al.*, 2006
BP100 and derivatives	Artificially designed	*Nicotiana benthamiana*	Transient expression via agroinfiltration	*CaMV35S*	Analysis of subcellular localization	ND	Company *et al.*, 2014
		Arabidopsis thaliana	Constitutive	*CaMV35S*	Transgenic plants failed to develop into maturity	ND	Company *et al.*, 2014
		Oryza sativa	Stable constitutive	Maize *ubiquitin-1*	PP against bacterial pathogens[1]	0.5% TSP	Company *et al.*, 2014[1]
		O. sativa	Stable constitutive	Maize *ubiquitin-1*	PP against *Dickeya chrysanthemi* and *Fusarium verticillioides*, Improved tolerance to oxidative stress[1]	ND	Nadal *et al.*, 2012[1]
Cathelicidin	LL-37, derivative of hCAP18 from human	*Brassica rapa*	Constitutive	*CaMV35S*	PP against bacterial and fungal diseases	ND	Jung *et al.*, 2012
CecA	Cecropin A from *Hyalophora cecropia*	*O. sativa*	Stable constitutive	Maize *ubiquitin-1*	PP against *Magnaporthe grisea*	ND	Coca *et al.*, 2006
		O. sativa	Endosperm specific	Rice *glutelin B1*, Rice *glutelin B4*	PP against *F. verticillioides* and *Dickeya dadantii*[1]	1–4 μg g^{-1} seeds	Bundó *et al.*, 2014[1]
CecB	Cecropin B from *Hyalophora cecropia*	*Solanum lycopersicum*	Stable constitutive	*CaMV35S*	PP against *Ralstonia solanacearum* and *X. campestris* pv. vesicatoria	~0.001 μg mg^{-1} leaves	Jan *et al.*, 2010

CecP1	Cecropin P1 from *Sus scrofa*	*Camelina sativa*, *B. napus*	Stable constitutive	*CaMV35S*	PP against *Erwinia carotovora* and *F. sporotrichioides*, Improved tolerance to salinity and oxidative stress	ND	Zakharchenko *et al.*, 2013a,b
		S. tuberosum	Stable constitutive	*CaMV35S*	PP against *Phytophthora infestans*, *Sclerotinia sclerotiorum*	ND	Zakharchenko *et al.*, 2007
CEMA	Cecropin A-melittin hybrid peptide, artifical	*N. tabacum*	Pathogen responsive	Poplar *win3.12T*	PP against *F. solani*	ND	Yevtushenko *et al.*, 2005
D4E1	Artificially designed	*Gossypium hirsutum*	Constitutive	*CaMV35S*	PP against fungal pathogens	ND	Rajasekaran *et al.*, 2005
Defensins	Defensin 02 from *N. megalosiphon*	*N. tabacum*, *S. tuberosum*	Constitutive	*CaMV35S*	PP against various bacterial and fungal pathogens	ND	Portieles *et al.*, 2010
	Wasabi defensin from *Wasabia japonica*	*Colocynthis citrullus* (Egusi melon)	Constitutive	*CaMV35S*	PP against *Alternaria solani* and *F. oxysporum*	ND	Ntui *et al.*, 2010
	Defensin 1 from *Medicago sativa*	*Lycobersicum esculentum Mill* (tomato)	Constitutive	*CaMV35S*	PP against *F. oxysporum*	ND	Abdallah *et al.*, 2010
	Defensin 1 from *B. rapa*	*O. sativa*	Stable constitutive	Rice *cytochrome C*	PP against a sap-sucking insect, *Nilaparvata lugens*	ND	Choi *et al.*, 2009
Dermaseptin	From *Phyllomedusa sauvagii*	*C. sinensis*	Constitutive	*CaMV35S*	PP against citrus canker caused by *X. axonopodis*	ND	Furman *et al.*, 2013

Dermaseptin and/or AP24 and/or lysozyme	Various combinations of dermaseptin from *P. sauvagii*, AP24 osmotin from *N. tabacum* and lysozyme from *Gallus gallus*	*S. tuberosum*	Stable constitutive	*CaMV35S*	PP against bacterial and fungal diseases, Gene stacking	ND	Rivero *et al.*, 2012
HT	Hordothionin from *Hordeum vulgare*	*Ipomoea batatas* (sweet potato)	Constitutive, Organ-specific, Sugar-inducible	Chimeric E12Ω (variant of *CaMV35S*), Potato *β-amylase*	PP against *Ceratocystis fimbriata*	ND	Muramoto *et al.*, 2012
		Apple	Constitutive	*CaMV35S*	PP against apple scab caused by *Venturia inaequalis*	ND	Krens *et al.*, 2011
Mag2 and derivatives	Magainin-2 from *Xenopus laevis* and a derivative of magainin-2 (MSI99)	*Vitis vinifera*	Stable constitutive	Arabidopsis *ubiquitin-3*	PP against *Agrobacterium vitis* and *Uncinula necator*	ND	Vidal *et al.*, 2006
	Derivative of magainin-2 (MSI99), artificial	*Musa* spp. (banana), *N. tabacum*	Stable constitutive	Arabidopsis *ubiquitin-3*	PP against bacterial and fungal diseases	ND	Chakrabarti *et al.*, 2003
Metchnikowin	From *Drosophila melanogaster*	*H. vulgare*	Pathogen-inducible	*A. tumefaciens mannopine synthase*	PP against *Blumeria graminis* and *F. graminearum*	ND	Rahnamaeian *et al.*, 2009
MsrA2	Derivative of dermaseptin B1 from *P. sauvagii* and *P. bicolor*, artificial	*N. tabacum*, *S. tuberosum*, Poplar	Pathogen responsive	Poplar *win3.12T*	PP against fungal diseases	ND	Yevtushenko and Misra, 2007; Yevtushenko and Misra, 2012
		S. tuberosum	Organ-specific	Douglas-fir *BiP Pro1-1*	Post-harvest PP against *Pectobacterium carotovorum*	8 μg g^{-1} fresh tuber tissue	Yevtushenko and Misra, 2012

MsrA3	Derivative of temporin A from *Rana temporaria*, artificial	*S. tuberosum*	Constitutive	*CaMV35S*	PP against *E. carotovora*, *Phytophthora infestans* and *Phytophthora erythroseptica*	ND	Osusky *et al.*, 2004
Pen4-1	Penaeidin4-1 from *Litopenaeus setiferus*	*Agrostis stolonifera* (creeping bentgrass)	Stable constitutive	Maize *ubiquitin-1*	PP against *Sclerotinia homoecarpa* and *Rhizoctonia solani*	ND	Zhou *et al.*, 2011
PG1	Protegrin-1 from porcine leukocytes	*N. tabacum*	Stable chloroplast	Tobacco *psbA*	PP against *E. carotovora*	17–26% of TSP	Lee *et al.*, 2011
Rev4	Reverse peptide of indolicidin isolated from bovine neutrophils, artificial	*N. tabacum*, *A. thaliana*	Stable constitutive	Peanut chlorotic streak caulimovirus (PClSV)	PP against *E. carotovora*, *Peronospora tabacina* and *Pseudomonas syringae*, Enhanced yield	ND	Xing *et al.*, 2006
Rev4 and Myp30	Rev4 and a derivative of magainin-2 (Myp30)	*A. thaliana*	Stable constitutive	*CaMV35S*	PP against *Pseudomonas syringae* and *Peronospora parasitica var Noco2* Gene stacking	ND	Xing *et al.*, 2006
RC101	Retrocyclin-101 based on *Macaca mulatta* minidefensins, artificial	*N. tabacum*	Stable chloroplast	Tobacco *psbA*	PP against *E. carotovora* and tobacco mosaic virus	32–38% of TSP	Lee *et al.*, 2011
RsAFPs	Antifungal proteins from *Raphanus sativus*	*Triticum aestivum*	Stable constitutive	Maize *ubiquitin-1*	PP against *F. graminearum* and *Rhizoctonia cerealis*	ND	Li *et al.*, 2011
		O. sativa	Stable constitutive	Maize *ubiquitin-1*	PP against *Magnaporthe oryzae* and *Rhizoctonia solani*	ND	Jha and Chattoo, 2010

SmAMPs	Hevein-like AMPs from seeds of *Stellaria media*	*A. thaliana*, *N. tabacum*	Constitutive	*CaMV35S*	PP against *Bipolaris sorokiniana* and *Thielaviopsis basicola*	ND	Shukurov *et al.*, 2012
Temporin A	From skin secretion of *Rana temporaria*	*N. tabacum*	Pathogen responsive	Poplar *win3.12T*	PP against fungal diseases	ND	Yevtushenko and Misra, 2007
Thanatin	From *Podisus maculiventris*	*O. sativa*	Constitutive	*CaMV35S*	PP against *Magnaporthe oryzae*	ND	Imamura *et al.*, 2010
Thionins	From *B. oleracea*, *Nasturtium officinale* and *Barbarea vulgaris*	*S. tuberosum*	Constitutive	*CaMV35S*	PP against *Botrytis cinerea*	ND	Hoshikawa *et al.*, 2012

[1] Potential use of the plant host and/or the expression system for molecular farming of therapeutic antimicrobial peptides has been indicated in the study.

1.3.1.1 Constitutive or inducible expression of antimicrobial peptides in plants

Strong and constitutive promoters that are frequently employed to drive AMP expression for plant protection include *cauliflower mosaic virus 35S RNA* (*CaMV35S*) promoter or *ubiquitin* promoters from various sources (Table 2). The undecapeptide BP100, which was identified from a library of synthetic cecropin A-melittin hybrids (Badosa *et al.*, 2007), was employed as an agent for plant protection (Company *et al*., 2014; Nadal *et al.*, 2012). Additionally, derivatives of BP100 were designed based on structural requirements for high-level expression in plants (Badosa *et al.*, 2013). Expression of BP100 and derivatives was achieved under the control of *CaMV35S* or *ubiquitin* promoters (Table 2). The *Raphanus sativus* antifungal protein 2 (RsAFP2) from radish, expressed under *CaMV35S* promoter, provided resistance to a fungal pathogen *Alternaria longipes* in transgenic tobacco (Terras *et al.*, 1995). The same defensin from radish, i.e., RsAFP2, was expressed in wheat under maize *ubiquitin-1* promoter. Transgenic wheat lines displayed enhanced resistance to *Fusarium graminearum* and *Rhizoctonia cerealis* (Li *et al.*, 2011).

On the other hand, use of organ-specific or inducible promoters to precisely control transgene expression offer clear advantages. Inducible poplar *win3.12T* promoter, which exhibited strong systemic activity upon pathogen challenge or wounding, was transcriptionally fused to the plant-optimized nucleotide sequences of either *MsrA2* or *temporin A* for development of disease resistance in tobacco (Yevtushenko and Misra, 2007). Transgenic plants had normal phenotype and were resistant to various pathogenic fungi. Moreover, lowest susceptibility to pathogens was observed in lines with highest transgene expression and AMP accumulation which were directly correlated to the gene copy number. In a bioassay with detached leaves, lines with multiple copies of the transgenes were found to be more tolerant to pathogens than those with a single copy (Yevtushenko and Misra, 2007). Similar correlation between transgene copy number, transcript accumulation and strength of disease resistance was reported in other studies (Ntui *et al.*, 2010; Yevtushenko *et al.*, 2005).

In another example of transgene expression under an inducible promoter, expression of an insect antifungal peptide (metchnikowin) was driven under the control of a pathogen-responsive *mannopine synthase* (*mas*) promoter in barley plants. Metchnikowin accumulated in plant apoplastic space in response to powdery mildew

as well as *Fusarium* head blight and root rot, and conferred resistance on transgenic barley lines (Rahnamaeian *et al.*, 2009). Tissue specific expression can also be used to avoid the phytotoxic effects of AMPs in vegetative tissues. In a recent study, rice *glutelin B1* or *B4* promoter sequences were used for endosperm specific expression of a bioactive peptide, cecropin A (Bundó *et al.*, 2014). Transgenic lines were resistant to *Fusarium verticillioides* and *Dickeya dadantii*, and the rice seeds accumulating cecropin A were viable. The amount of accumulated peptide was also determined by an immunoblot assay of protein extracts from transgenic mature seeds, and it was indicated that the study has implications for both molecular farming and plant protection (Bundó *et al.*, 2014). In another study, tuber specific expression of a dermaseptin B1 derivative MsrA2 was driven by *BiP Pro1-1* promoter from Douglas-fir, and heterogeneous AMP enabled engineering of resistance to soft rot caused by pathogenic *Pectobacterium carotovorum* in potato plants (Yevtushenko and Misra, 2012).

1.3.1.2 Strategies to enhance stability of antimicrobial peptides

Evidence on absence of pathogen resistance in transgenic plants expressing AMPs was also reported in couple of studies. Peptides with *in vitro* biocidal properties did not provide resistance to pathogens when they were expressed in plants (Florack *et al.*, 1995; Hightower *et al.*, 1994). One of the possible explanations for low disease resistance is the cellular degradation of foreign AMPs by plant endogenous proteases (Mills et al., 1994). Stability and accumulation levels of peptides are critical for generation of antimicrobial response during pathogen invasion. Hence, researchers employ diverse strategies to achieve stability and accumulation. It has been proposed that one of the most crucial factor influencing not only *in vivo* stability of the recombinant peptide, but also the final yield is subcellular targeting of the product.

The most commonly used strategy to enhance stability is inclusion of auxiliary signal sequences from source, host or closely related organisms to target the product to extracellular space (Boscariol *et al.*, 2006; Bundó *et al.*, 2014; Chakrabarti *et al.*, 2003; Coca *et al.*, 2006; Jan *et al.*, 2010; Rivero *et al.*, 2012; Vidal *et al.*, 2006; Xing *et al.*, 2006; Zhou *et al.*, 2011). Most of the proteins or peptides lacking a signal

peptide accumulate in the cytosol, generally resulting in low yields (Conrad and Fiedler, 1998). Furthermore, since phytopathogenic microorganisms multiply in the apoplast before attacking plant cells (Alfano and Collmer, 1996), secretion of AMPs should enhance peptide-pathogen interaction and prevent tissue colonization by pathogens (Düring, 1996). For instance, Boscariol *et al.* (2006) used successfully an insect native signal peptide for secretion of the insect-derived *attacin A* gene product to intercellular space in transgenic sweet orange in order to obtain plants resistant to *Xanthomonas axonopodis*, the causative agent of citrus canker. In another study, *cecropin B* gene from giant silk moth was fused to barley α-amylase signal sequence, and the chimeric gene was used for constitutive expression of cecropin B in tomato (Jan *et al.,* 2010). Transgenic plants were shown to be significantly resistant against bacteria wilt and bacteria spot, diseases caused by two of the major pathogens of tomato.

Nevertheless, targeting to apoplastic space is not always necessary to obtain disease resistance (Abdallah *et al.*, 2010; Imamura *et al.*, 2010; Jha and Chattoo, 2010; Li *et al.*, 2011; Osusky *et al.*, 2004). Transformation of creeping bentgrass was performed using two different constructs containing either the coding sequence of penaeidin4-1 from shrimp or the penaeidin4-1 fused to secretion signal sequence from tobacco AP24 protein (Zhou *et al.*, 2011). Transgenic lines exhibited similar levels of resistance to brown patch and dollar spot regardless of the construct used for their transformation.

In another study, constitutive expression of *cecropin A* gene designed to secret the encoded peptide to extracellular space had negative effects on fitness and fertility of transgenic rice plants, whereas cecropin A, which had an extra C-terminal KDEL tetrapeptide extension and was retained in ER, had no phenotypic effects on transgenic plants (Coca *et al.,* 2006). In general, stability of recombinant peptide is largely influenced by its final subcellular destination. Native seed protein storage organelles (such as ER-derived protein bodies and *de novo* formed protein storage vacuoles) offer tremendous benefits in terms of product protection from degradation. Additionally, protein storage bodies aid in purification steps as well as post-harvest encapsulation. Expression of an AMP with certain protein fusion partners, which induce aggregation of protein bodies-like organelles (even in plant tissues normally lacking such subcellular compartments) might result in peptide accumulation in a practical and efficient manner.

Examples of such fusion partners include natural zeins, elastin-like polypeptides and hydrophobins (Khan *et al.*, 2012).

Fusion of a target AMP to a carrier protein tag is regarded as an effective strategy to stabilize the peptide, increase its accumulation, and protect the final product from proteolytic degradation. Moreover, protein fusions can mask the lethal effects of AMPs on host plant cells (Viana *et al.*, 2013). Bioactive defense peptides were expressed in hairy roots of tomato for development and evaluation of a strategy to reduce infection caused by pathogenic organisms targeting tomato roots (Fang *et al.*, 2006). The peptides were fused at C-terminus with a secreted enzyme from maize, which acted as a display scaffold. The peptide–scaffold fusion products accumulated in the rhizosphere and reduced pathogen infection significantly (Fang *et al.*, 2006). On the other hand, the carrier protein in the peptide–scaffold construct might retain its enzymatic activity and expression of a phytohormone-degrading enzyme in intact plants might have negative effects on plant development as a result of a probable phytohormone imbalance (Fang *et al.*, 2006). In addition to the aforementioned carrier protein, there are various tags widely used in heterologous expression of peptides. They can be grouped according to their common features and include easy to detect fusions, fusions to viral coat proteins, immunogenic protein partners, and purification-facilitating proteins (Viana *et al.*, 2013).

1.3.2 Therapeutic antimicrobial peptide production in plants

Recent advances in biotechnology allowed use of plant bioreactors as attractive platforms for commercial production of various peptides, proteins and pharmaceuticals. High-level product expression and accumulation as well as recovery require consideration of certain features of the product and employment of specific strategies, as discussed in the following sections.

1.3.2.1 Expression of therapeutic peptides in plant chloroplasts

There are several possibilities, including transient or stable transformation of nuclear or chloroplast genomes of host plants, for heterologous expression of AMPs. Among these, transformation of chloroplast genomes, i.e., generation of transplastomic plants, are considered prospective and efficient for functional expression of AMPs at high levels. Up to 20,000 copies of the transgene per cell can be expressed after gene integration into the chloroplast genome. Production of AMPs, retrocyclin-101 and protegrin-1, which have complex secondary structures stabilized by disulphide bridges, have been achieved by chloroplast transformation (Lee *et al.*, 2011). Both peptides cannot be produced in microbial systems because of their complex structures and antimicrobial activities. Retrocyclin-101 protects human cells from infection by HIV type 1 (Cole *et al.*, 2002) and retains its full activity in human cervicovaginal tissue (Cole *et al.*, 2007), whereas protegrin-1 is potent in inactivating *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, two causal agents of sexually transmitted diseases in human (Qu *et al.*, 1996; Yasin *et al.*, 1996). When expressed with a green fluorescent protein (GFP) fusion and a histidine tag in tobacco chloroplasts, biologically active retrocyclin-101 and protegrin-1 accumulated up to 38% and 26% of total soluble protein, respectively. It was also indicated that organic extraction of retrocyclin-101 resulted in almost 11-fold higher yield compared to affinity purification using the histidine tag (Lee *et al.*, 2011).

1.3.2.2 Transient expression of antimicrobial peptides using viral vectors

Recently, a production system that was based on tobacco mosaic virus (TMV) and use of the full virus as a vector was developed for transient expression of a *de novo* designed peptide SP1-1 in *Nicotiana benthamiana* plants (Table 3). Viral coat protein used as a fusion partner enabled presentation of the AMP on the surface of viral particles (Zeitler *et al.*, 2013). The amphipathic peptide SP1-1 is active against both plant and human pathogens, including multi-resistant *Staphylococcus aureus* (Dangel *et al.*, 2013). After successful production and subsequent extraction of recombinant virions, the SP1-1 peptide was cleaved off from the fusion partner by bromocyanide, and the target peptide was purified using chromatographic methods. It was indicated

that virus-based transient expression of SP1-1 yielded up to 0.025 mg of pure, biologically active AMP per g of leaf biomass (Zeitler *et al.*, 2013).

Taken together, published data demonstrate that establishment of viable transgenic plants expressing bioactive AMPs represent a promising strategy for therapeutic peptide production as well as crop improvement. However, there is no universal expression strategy. Systematic approaches need to be employed for individual peptides and plant hosts depending on their particular properties.

Table 3: Selected list of antimicrobial peptides expressed in plants for plant molecular farming (PMF). ND: not determined.

Peptide name	**Description and peptide origin**	**Plant host**	**Expression system and strategy**	**Promoter**	**Application**	**Yield**	**References**
Cathelicidin	SMAP-29 from sheep myeloid cells	*Nicotiana tabacum*	Constitutive	*CaMV35S*	PMF	ND	Morassutti *et al.*, 2002
GLP-1	Glucagon-like peptide 1 from human	*Oryza sativa*	Endosperm-specific	Rice *glutelin B1*	PMF, Potential use as a therapeutic agent of type II diabetes	ND	Yasuda *et al.*, 2005
Interferons	Interferon-α1 from *Salmo salar* (SasaIFN-α1)	*Solanum tuberosum*, *O. sativa*	Constitutive	*CaMV35S*	PMF, Disease prevention in fish against viral infections	ND	Fukuzawa *et al.*, 2010
	Interferon-α from chicken (ChIFN-α)	*Lactuca sativa*	Transient expression via agroinfiltration	*CaMV35S*	PMF, Viral disease prevention in chicken	0.393 μg kg^{-1} tissue (0.0004% TSP)	Song *et al.*, 2008
Lactostatin	Derived from β-lactoglobulin in cow's milk	*O. sativa*	Endosperm-specific	Rice *10-kDa prolamin*, Rice *glutelin B4*	PMF using cereal seeds, Potential clinical use as an anti-hypercholesterolemic peptide-based drug	2 mg g^{-1} dry seeds	Cabanos *et al.*, 2013
MsrA2	Derivative of dermaseptin B1 from *Phyllomedusa sauvagii* and *P bicolor*, artificial	Poplar	Constitutive	*CaMV35S*	PMF	2–6 μg g^{-1} fresh mass	Yevtushenko and Misra, 2012
rC4V3	V3 loop fused to C4 domain from gp120 of human immunodeficiency virus (HIV), artificial	*N. tabacum*	Stable chloroplast	*Prrn*	PMF, Induction of mammalian immune response against HIV, Potential HIV vaccine	ND	Rubio-Infante *et al.*, 2012
SP1-1	*de novo* designed, artificial	*N. benthamiana*	Transient	*SP6*	PMF, Potential use against both plant and human pathogens	0.025 mg g^{-1} leaf mass	Zeitler *et al.*, 2013

1.4 Plant-based expression strategies for antimicrobial peptide production

As stated in previous sections, AMP production in plants is employed for two main purposes, namely plant protection and molecular farming. Various plant-based expression platforms ranging from cultures of cells or tissues to transgenic plants offer diverse tools for these two applications. Plant-based systems provide cost-effective production and exhibit high scale-up capacity (Fig. 5). These systems are considered promising since they address drawbacks of other biological expression systems (Basaran and Rodriguez-Cerezo, 2008). The main advantages of plant-based systems include high product yield, quality and homogeneity obtained in these systems. Proper folding, glycosylation and disulphide bond formation, which are critical for AMP activity, can also be achieved in plant-based systems (Ma *et al.*, 2003; Ramessar *et al.*, 2008). When expression systems are compared according to costs associated with development, bacterial expression platforms are more desirable (Fig. 5) since bacteria and yeast are easy to transform and maintain while plant-based systems require more effort, time and cost. However, once established plant-based systems offer cost-efficient production, easy, fast and high-capacity scale-up, and low-cost purification and storage (Fig. 5), all of which rank plant-based platforms more desirable among other biological systems.

Various heterologous expression strategies have been used to produce AMPs in large-scale. Among these strategies, microbial systems, i.e., bacteria and yeast, have been the most widely employed hosts for peptide expression (Parachin *et al.*, 2012). However, AMP expression in microorganisms might hinder microbial growth or decrease expression level of the target peptide. Thus, microbial expression of an AMP requires controlled or induced transcription and/or fusion to a carrier peptide or protein. Plants, on the other hand, present a suitable alternative for production of AMPs without the need for controlled expression. Furthermore, plant-based platforms are considered safe since plants are free of animal or human pathogens and endotoxins, and there is almost no risk of product contamination (Daniell *et al.*, 2001; Fischer *et al.*, 2004; Magnusdottir *et al.*, 2013). Although risk of contamination and therapeutic risks are low in plant-based expression systems, public perception of risks are high. This misconception ranks plant-based systems, specifically GM plants, among the least desirable in biological expression

platforms (Fig. 5). Thus, effort should be invested in public education to build a comprehensive and accurate view on use of GM plants for molecular farming.

Although a wide range of plant-based platforms for AMP production is available, certain stages of production process and parameters affecting these stages should be considered attentively while choosing a platform for AMP molecular farming in plants. Yield of heterologous AMP, cost and time required for production, market size of the product, production scale and capacity of the process, and downstream processing should be analysed in detail for successful applications in molecular farming of AMPs in plant-based systems. Time required for transgenic plant development and biosafety concerns related to GM plants are the main disadvantages in plant-based systems (Fig. 5).

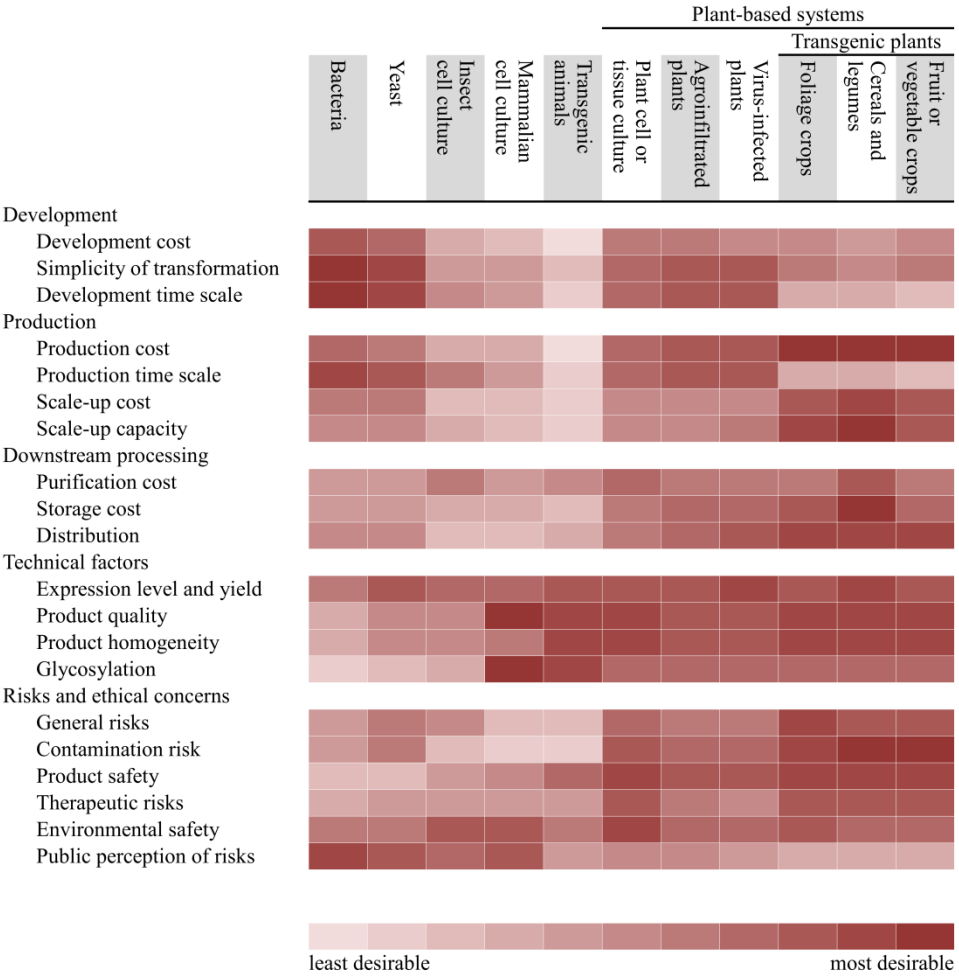

Figure 5. Comparison of heterologous expression systems for production of biological molecules. Coloring is used to rank expression systems from least to most desirable according to various parameters and properties. Adapted from Daniell *et al.* (2001), Fischer *et al.* (2004), Ma *et al.* (2003), and Parachin *et al.* (2012).

1.4.1 Whole transgenic plants

Stable constitutive expression of peptides in whole plant body can be achieved with various technologies for transgene integration to plant genome. Transgenic plants which contain the transgene in their genome over many generations are considered stable, and allow easy scale-up and low-cost production. In most of the studies, constitutive expression of AMPs was achieved under *CaMV35S* or *ubiquitin* promoters (Abdallah *et al.*, 2010; Jan *et al.*, 2010; Portieles *et al.*, 2010; Rivero *et al.*, 2012; Shukurov *et al.*, 2012; Vidal *et al.*, 2006; Zhou *et al.*, 2011).

Expression in whole plant body can be employed for development of disease resistance and/or crop improvement. Potato plants have been transformed with alfalfa antifungal peptide (Gao *et al.*, 2000) and tachyplesin I from horseshoe crab (Allefs *et al.*, 1996), which provided various levels of protection against fungal pathogen *Verticillium dahlia* and bacterial pathogen *Erwinia carotovora*, respectively. In another study, constitutive expression of cecropin P1 in transgenic potato conferred resistance to the fungal pathogens *Sclerotinium sclerotiorum* and *Phytophthora infestans*, the causative agents of white rot and potato blight (Zakharchenko *et al.*, 2007). Constitutive expression of natural or synthetic AMPs under *CaMV35S* or maize *ubiquitin-1* promoters has also been employed for molecular farming (Company *et al.*, 2014; Nadal *et al.*, 2012).

However, constitutive expression and subsequent accumulation of an AMP might negatively affect biological functions in a host plant (Coca *et al.*, 2006) and lead to undesirable selection pressure on pathogenic microorganisms. Recently, constitutive expression of tandemly arranged units of a synthetic peptide BP100 was shown to be toxic for rice and *Arabidopsis* (Company *et al.*, 2014; Nadal *et al.*, 2012). Toxicity was avoided by expression of inverted repeats of BP100 whether or not elongated with sequences derived from natural AMPs. It was also indicated that the complex equilibrium between AMP phytotoxicity, antimicrobial activity and transgene-derived plant stress response is critical for fitness and disease resistance of GM plants constitutively expressing AMPs (Company *et al.*, 2014; Nadal *et al.*, 2012).

Certain wound- or pathogen-inducible promoters which were used for development of disease resistance in plants offer controlled expression, help eliminate phytotoxic effects of AMPs in host plants, and avoid unintended effects

on non-target organisms interacting with plants (Rahnamaeian *et al.*, 2009; Yevtushenko *et al.*, 2005). Besides transcriptional control with promoters, product yield can also be boosted in many plant host systems with strategies at various stages of product synthesis, including translation, post-translational modification and subcellular targeting (Makhzoum *et al.*, 2014; Obembe *et al.*, 2011). Most widely used translational fusions include signal sequences for apoplastic secretion, ER retention and intracellular targeting. Additionally, fusion to certain purification tags, such as hexameric histidine tag and maltose binding protein, might also stabilize the product and help boost the expression.

1.4.2 Tissue specific expression

Although heterologous expression in whole plant body enables high-level accumulation of recombinant product, the proteins or peptides expressed in all tissues such as leaves, pollen and roots might adversely affect growth and development of the transgenic plants. Expression in all plant parts could also expose herbivores and pollinating insects to the effects of the recombinant peptide, actuate undesired development of resistance among pathogens, and lead to unfavorable environmental consequences. Additionally, various tissues and their complex composition may exert hurdles during isolation and purification of the target AMP. Restriction of peptide accumulation to certain tissues using tissue specific promoters can help reduce these risks. Therefore, desired expression level and functional stability of the product as well as spatial and temporal accumulation should be considered during establishment of stable transgenic plants. Tissue specific expression of peptides might overcome the problems associated with expression and stability of the product as well as downstream processing.

Main advantages of tissue specific expression include, but not limited to, contained expression, elimination or relief of AMP cytotoxicity in host plant during certain developmental stages and avoidance of undesired environmental effects. Additionally, AMP stability might be improved and downstream processing and peptide purification might be simplified via expression in certain tissues (Xu *et al.*, 2012). Grains of cereals and legumes provide inert and stable environment for peptide accumulation and storage (Lau and Sun, 2009). Furthermore, diverse seed specific

promoters not only in barley, but also in wheat, maize, bean and others have been identified (De Jaeger *et al.*, 2003; Mrízová *et al.*, 2014; Ramessar *et al.*, 2008). In numerous studies, rice *prolamin* and *glutelin* promoters (Bundó e*t al.*, 2014; Cabanos *et al.*, 2013; Takagi *et al.*, 2005; Yasuda *et al.*, 2005), barley *hordeinD*, *α-amylase*, and *trypsin inhibitor* promoters (Joensuu *et al.*, 2006; Stahl *et al.*, 2002), wheat *glutenin* promoter (Schünmann *et al.*, 2002) and soybean *glycinin* promoters (Hudson *et al.*, 2014; Moravec *et al.*, 2007) have been employed for expression of various peptides and proteins in grains. These studies generally have implications towards molecular farming of pharmaceutical peptides or proteins, since grains are cheap to produce and harvest, easy to store and distribute, and might be employed as edible vaccines. For example, canola seeds were used for accumulation of a recombinant protein, hirudin that was stable in dry seeds for over three years (Boothe *et al.*, 1997). In another study, endosperm specific expression of lactostatin, a peptide with hypocholesterolemic activity, was achieved in rice. Twenty nine copies of *lactostatin* sequences were inserted into the gene sequence of non-conserved regions of a soybean seed storage protein (A1aB1b), and the resulting chimeric gene was used for stable transformation of rice. Transgenic lines accumulated approximately 2 mg of lactostatin per g of dry seeds (Table 3; Cabanos *et al.*, 2013). On the other hand, strict regulations related with GM crops should be considered carefully, and cultivation, harvest and distribution of these crops, specifically GM cereals and legumes, should be isolated from that of unmodified crops, as they provide the main food and feed groups for human and animals.

Besides molecular farming, expression restricted to a certain tissue might also be employed for plant protection. Certain phytopathogens attack plants at certain tissues, such as roots, tubers and leaves. Development of disease resistance at the site of attack is critical for durable resistance and sustainable productivity. Additionally, tissue or organ specific accumulation of AMPs might reduce post-harvest loss in crop plants (Yevtushenko and Misra, 2012).

1.4.3 Expression strategies that utilize plant cell or tissue culture

There are different types of *in vitro* cultures where plant cells or tissues are maintained using a well-adjusted balance of phytohormones. Among them, cultures of suspension cells, calli and hairy roots represent well-established techniques, in which plant cells or tissues are cultivated under specific conditions absolutely independent of climatic or geographic factors. Common features of these *in vitro* cultures include simplicity of transformation, product homogeneity and the short period of time required for accumulation of a heterologous product, which is generally days or weeks after transformation. *In vitro* cultivation methods are also considered powerful tools for testing diverse expression strategies before they are further employed for generation of stable transgenic plants. Moreover, these cultivation techniques are desirable for evaluation of secretion signal sequences, since recombinant products can easily be recovered and purified from the culture medium without large quantities of contaminating macromolecules such as proteins and carbohydrates (Georgiev *et al.*, 2007; Miao *et al.*, 2008; Plasson *et al.*, 2008).

Callus cultures can be obtained from almost every living plant tissue, although young tissues and tissues with meristematic or promeristematic activities are preferred (Petersen and Alfermann, 2008). Transgenic callus cultures are generally obtained via direct transformation of calli (Hiei and Komari, 2008), or via transformation of explants and subsequent callus induction from these explants (Carciofi *et al.*, 2012; Imani *et al.*, 2011). Although cultured calli are not commonly used for production of heterologous compounds, they can be employed as potent testing platforms especially for plants, where time-efficient and robust transformation screening technologies are not established. Most of monocotyledonous crop plants are recalcitrant to transformation or *in vitro* regeneration and/or lack a high-throughput screening platform for evaluation of expression strategies. Hence, callus cultures represent alternative screening tools or models which provide identical physiological and genetic background as that of the host in the target expression platform. In a recent study, a probable use of transgenic barley callus as a model system for evaluation of transgenic modification strategies for starch bioengineering in cereals was reported. It was indicated that within 9 weeks, as much as 1.5 g dry weight of fully transgenic calli generated after *Agrobacterium*-mediated transformation of barley immature embryos could be obtained (Carciofi *et al.*, 2012). In another study, a system called STARTS for generation

of stable transgenic roots from barley calli was developed. The strategy, which allowed analyses within 6 weeks, was employed for functional analysis of an expressed barley protein (Imani *et al.*, 2011).

Interest in plant cell suspension cultures as expression platforms has increased over the last decade. Undifferentiated cultured cells of plants bear most of the benefits offered by whole plants and exhibit advantages of microbial systems, such as easy manipulation. Suspension cultures of plant cells are composed of relatively homogeneoussuspensions of rapidly dividing cells established by transfer of callus (either transgenic or not) into liquid media. Suspension-cultured transgenic plant cells can also be prepared by their direct transformation (King, 1984; Xu *et al.*, 2011). The most frequently employed plant species for recombinant product expression in suspension cultures is tobacco, since tobacco cells are easy to transform and maintain, and multiply rapidly. On the other hand, various other plant species have been utilized for establishment of cell cultures, including rice, soybean, alfalfa, and tomato (Xu *et al.*, 2011). To date, diverse bioactive pharmaceutical compounds including those with antimicrobial properties have been produced in suspension cultures. A functional human lysozyme was expressed in transgenic rice cell cultures, and expression levels of the recombinant lysozyme reached approximately 4% of total soluble protein (Huang *et al.*, 2002). In another study, tobacco Bright Yellow-2 (BY-2) cells were used for characterization of expression and activity of a peptide (ACHE-I-7.1) mimetic of aldicarb, an effective carbamate nematicide. After confirmation of biological activity of the peptide in suspension cultures, the construct encoding ACHE-I-7.1 was used for generation of transgenic potato plants that exhibited increased resistance to root nematode invasion (Liu *et al.*, 2005). Although suspension cell cultures do not offer scale-up capacity as high as transgenic plants, confined expression in bioreactors allow precise control over production process, and provide a platform accordant with regulations and regarded as environmentally and therapeutically safe. Various biologically active pharmaceutical proteins have successfully been produced in plant cell cultures. The first commercially available therapeutic protein, that is manufactured using a plant-based system, is actually produced in carrot root cell suspension cultures (Shaaltiel *et al.*, 2007). This plant-made recombinant protein (glucocerebrosidase), formulated as Elelyso™ (Protalix Biotherapeutics Inc., Israel), has been approved by the US Food and Drug Administration (FDA) in May 2012 for enzyme replacement

therapy of Gaucher's disease (Aviezer *et al.* 2009; Grabowski *et al.* 2014). Production of glucocerebrosidase in carrot cell suspensions provides cost-efficient and pathogen-free manufacturing compared to counterparts produced in Chinese hamster ovary cells or mammalian cell cultures. Additionally, counterparts require post-production modifications in product glycosylation to expose terminal α-mannose residues, which are needed for mannose receptor-mediated uptake by target cells. These modifications increase production cost. Plant-made glucocerebrosidase, also called taliglucerase alfa, requires no additional steps to yield a recombinant enzyme with exposed terminal mannose residues (Grabowski *et al.* 2014). Approval and commercial success of this drug will lead a new era in production of pharmaceuticals, including AMPs, in plant-based platforms. Major disadvantages of suspension cultures are their relative instability, low protein productivity and limited scale-up capacity. Moreover, cell cultures are very susceptible to contamination and overgrowth by microbes. Therefore, once transgenic suspension culture is established, it is desirable to prepare stocks in the form of callus cultures (Petersen and Alfermann, 2008; Xu *et al.*, 2011). Efficiency of different stages of production including genetic manipulation, gene expression, culturing, process development, product purification and downstream processing should be maximized in a systematic strategy to increase productivity in suspension cultures (Xu *et al.*, 2012). In a recent study on comparison of various plant-based expression platforms, transgenic cell suspension cultures were suggested as the most promising system for further optimization and large-scale production (Vasilev *et al.*, 2014). Hence, plant cell suspension cultures hold considerable promise for efficient production of therapeutic AMPs for medical approaches.

Hairy root systems are employed in a wide breadth of biotechnological applications such as therapeutic protein production, synthesis of phytochemicals and biotransformation of exogenous substrates (Banerjee *et al.*, 2012; Ono and Tian, 2011). Hairy root cultures are established by infection of plant cells or tissues with *A. rhizogenes*. Root inducing plasmid of *A. rhizogenes* is responsible for stable incorporation of genetic material (including the gene of interest) into the genome of a host plant cell. Generally, one to four weeks after successful transformation of the donor plant tissue, neoplastic roots start to grow in a highly branched manner with abundant lateral roots (Pavlov *et al.*, 2002; Sevón and Oksman-Caldentey, 2002). Hairy root cultures can also be employed for rapid evaluation of peptide activity

as well as efficiency and yield of peptide production in plant-based platforms. Although transgene silencing is a common feature observed in hairy root gene expression strategies (Sivakumar, 2006), long-term genetic and biosynthetic stability (in contrast to undifferentiated cell suspension cultures) together with the fact that more than 400 plant species have already been used for establishment of hairy root cultures (Porter, 1991) make expression in hairy root cultures an attractive tool for rapid assessment of diverse aspects of gene expression (Miao *et al.*, 2008; Ron *et al.*, 2014). Hairy root cultures were employed to demonstrate defensive role of AMPs in disease-susceptible tissues of plants. Transgenic potato hairy roots, expressing a levamisole-mimetic synthetic peptide (LEV-I-7.1), were employed to investigate the effects of LEV-I-7.1 on number of *Globodera pallida* nematodes that were able to establish in hairy roots. Transgenic hairy roots displayed approximately 50% reduction in the number of obligate root parasites (Liu *et al.*, 2005).

1.4.4 Alternative approaches employing transient expression

Among plant-based expression platforms, transient expression employing *Agrobacterium* or virus infection attracts attention due to short time scale for development and production, high expression levels and yields obtained, and confined nature of the system. In transient expression process, gene of interest is introduced into plant cells or tissues using an engineered vector, generally *Agrobacterium* or a plant virus, and protein or peptide production is achieved via extrachromosomal gene expression within plant cells. Heterologous expression starts within one day and lasts for several days to weeks depending on the host, product and the vector employed. During this process host cells or tissues, or plants themselves are maintained in contained environments such as greenhouses or bioreactors. After product purification the host is discarded and a fresh batch of untransformed host is prepared for a second round of production.

All transient expression systems use natural infective properties of *Agrobacterium* and plant viruses. Various approaches employing intact leaves, cultured hairy roots or suspension cells have been employed for expression of products in transient systems. Most of the studies used transient systems for evaluation of gene constructs, validation of activity of heterologous product or to determine efficiency

of expression platforms (Ben-Amar *et al.*, 2013; Circelli *et al.*, 2010; Company *et al.*, 2014). On the other hand, transient systems have also been used for production of industry-grade pharmaceutical proteins or peptides. In transient systems based on virus infection, various proteins and peptides including bovine lysozyme, human interleukin-2, human α-galactosidase A, bovine aprotinin and others have been produced (Pogue *et al.*, 2010). Among these, aprotinin is a 58 amino acid long polypeptide with serine protease inhibitor activity. Functional recombinant aprotinin was produced using tobacco plants infected with a TMV-based vector in greenhouses or field. The recombinant aprotinin exhibited properties highly comparable to an FDA-approved counterpart. Although it does not possess antimicrobial activity, production of aprotinin in a transient platform provides an example for possible expression of AMPs in transient systems for large-scale molecular farming. In another approach, efficient DNA delivery by *Agrobacterium* was combined with rapid replication and high level expression of a plant virus (Gleba *et al.*, 2005; Marillonnet *et al.*, 2003). Plant viral vector carrying gene of interest was introduced into intact tobacco plants via *Agrobacterium* vacuum infiltration. Various heterologous proteins including cytokines, interferons, bacterial and viral antigens, and growth hormones have been produced using this transient system (Giritch *et al.*, 2006; Gleba *et al.*, 2007). The approach has also been used for transient expression of an AMP, protegrin-1, in leaves of *N. tabacum* (Patino-Rodriguez *et al.*, 2013). Transgene expression and peptide accumulation in tobacco leaves were demonstrated 10 days post-infection. Additionally, *in vitro* assays of protein extracts from transiently transformed tobacco showed inhibitory activity of protegrin-1 on growth of various mammalian pathogens including *Klebsiella pneumonia*, *Staphylococcus aureus*, *Candida albicans* and others (Patino-Rodriguez *et al.*, 2013). In an attempt to adapt this transient system to large-scale manufacturing formats, viral vectors and *Agrobacterium* infiltration methods were improved (Pogue *et al.*, 2010). Traditional *Agrobacterium* vacuum infiltration was performed using automated conveyors and vacuum-rated large chambers. In this robust, large-scale process, the conveyors, loaded with trays holding intact tobacco plants rotate 180° and enter the chamber. The plants are submerged in *Agrobacterium* solution and vacuum is applied to aid bacterial infection. Plants, removed from chambers and rotated to upright position, are transferred to greenhouses for growth and product accumulation. After a growth period up to two weeks, plants are harvested, and product purification

is performed (Gleba *et al.*, 2005; Pogue *et al.*, 2010). A similar approach using transient expression in tobacco leaf tissues was employed by Medicago Inc., a Canada-based biopharmaceutical company, for production of a vaccine against avian H5N1 influenza. The vaccine has been used in a phase I clinical trial (Landry *et al.*, 2010). Overall, it is apparent that advantages offered by viral vectors, *Agrobacterium* infection and plant-based expression systems might all be combined in a transient, time- and cost-efficient, large-scale manufacturing process for molecular farming of pharmaceuticals.

Recently, a new approach named cell pack technology has been developed for production of pharmaceuticals, recombinant proteins and/or secondary metabolites. The technology combines the efficiency of methods based on transient expression via *Agrobacterium* or virus infection, with the advantages of plant cell suspension cultures. This high-throughput, cost-efficient and rapid platform makes use of cells from any plant cell suspension culture (e.g. *N. tabacum*, *A. thaliana*, *N. benthamiana*, *Catharanthus roseus*, *Daucus carota*). Initially, a medium-deprived, porous structured artificial tissue, called a cell pack or a cell cake, is generated. Cell packs are prepared by removal of liquid media from cell suspensions using simple filters. Subsequently, the cells are transformed, stably or transiently, by application of drops or spray of *Agrobacterium* suspension. Transformed cell packs are incubated one to six days under specific conditions in trays or columns until product recovery. Accumulated product can be harvested by a buffer solution, which allows elution of secreted proteins from packed cells. This rapid technology overcomes the problems associated with handling of large volumes of medium and buffers during production and downstream processing. Contrary to systems in liquid cultures, there is no need for control of bacterial growth, and the transgene is expressed more rapidly due to higher transformation efficiency. Furthermore, silencing triggered by an individual cell does not spread systematically. The method also provides easier product purification since there are less secondary metabolites and host proteins in packed cells compared to leaf-based expression systems. The technology was used for production of different antibodies translationally fused to various signal sequences or tags (Rademacher, 2013; Rademacher et al., 2019).

1.5 Tools for screening and evaluation of plant-based expression strategies

Although there are diverse heterologous expression systems for production of AMPs, particular properties of these peptides, such as size, folding and glycosylation should be considered carefully during selection of a host platform. Additionally, AMPs exhibit intrinsic instability, low immunogenicity, short half-life and certain level of cytotoxicity to host organism. Hence, researchers have been forced to develop specific expression strategies to address the limitations related to the target peptide and the host organism. These strategies mainly include, but not limited to, fusion of nucleotide sequence of the peptide to sequences of a proper promoter, a carrier protein, purification tags and/or subcellular targeting signals. Additionally, the transgene constructed for peptide production is generally subjected to codon optimization for high level expression in a certain host. All these modifications might alter the activity and stability of the target peptide as well as the yields obtained from an expression system. On the other hand, stable transformation of certain hosts, specifically plants, is a laborious and slow process. Therefore, efforts in the last few decades have been dedicated to establishment of rapid screening platforms for evaluation of efficiency and yield of expression strategies before they are employed for generation of stably transformed plants. These screening platforms generally employ transient expression of the target peptide in a well-established host. Selection of a screening technology is further critical since a distinct host with a different physiological and genetic background than that of the host in the target production platform might lead to deceptive results. This section provides an overview of the most potent plant-based screening systems or tools with specific emphases on time scale, cost, simplicity of transformation and suitability for diverse plant species.

1.5.1 Agroinfiltration of leaf tissues

Infiltration of intact leaf tissues with a suspension of *Agrobacterium tumefaciens* harboring an expression construct represent nowadays the most commonly used strategy for testing new constructs and generating valuable data. This labor-efficient, routine and cost-effective transient expression assay, with high transformation efficiencies, provide data with the analysis performed in several days without a need for selection

pressure on leaf tissues. Majority of the analysis in literature have been performed using *N. benthamiana* leaves (Sparkes *et al.*, 2006; Yang *et al.*, 2000). The procedure has also been optimized for other plant species including lettuce, tomato, *Arabidopsis* and grapevine (Ben-Amar *et al.*, 2013; Joh *et al.*, 2005; Tsuda *et al.*, 2012; Wroblewski *et al.*, 2005). In a recent study, production and subcellular localization of a synthetic AMP was demonstrated using a fluorescent tag in a transient expression system employing agroinfiltration. Western blot analysis of proteins extracted from *N. benthamiana* leaves three days after infiltration clearly confirmed the presence of the recombinant product in protein bodies derived from ER (Company *et al.*, 2014).

1.5.2 Seedling transformation

Use of young intact plant seedlings as hosts for transient transformation exhibits a big advantage in terms of time and space otherwise required for generation of mature transgenic plants. High levels of product expression can be achieved in 4 to 6 days in one week old or younger seedlings. In contrast to leaf infiltration techniques, use of seedlings overcomes the problem associated with diverse developmental stages of the leaves from the same donor plant, which might affect transformation efficiency. Several methods for transient transformation of seedlings have been described. In one of the earliest studies, young tobacco seedlings were used for expression of a marker gene after vacuum-infiltration in *Agrobacterium* suspension (Rossi *et al.*, 1993). McIntosh *et al.* (2004) developed an efficient and versatile transient assay system based on vacuum-infiltration of two week old *Arabidopsis* seedlings. A high level of reporter gene expression was observed five days after infiltration. This procedure was broadly followed and optimized in another study, where 3 to 4 days old seedlings were used as hosts, allowing the whole assay to be completed within one week (Marion *et al.*, 2008). Vacuum application is not always necessary to achieve accumulation of recombinant products in plant seedlings. Transient transformation has also been performed by simple co-cultivation of *Arabidopsis* seedlings with *Agrobacterium rhizogenes* (Campanoni *et al.*, 2007). In 2009, a fast *Agrobacterium*-mediated seedling transformation based on incubation of *Arabidopsis* seedlings with *A. tumefaciens* in the presence of a surfactant was reported (Li *et al.*, 2009). The assay was performed within one week from seed sowing to product analysis. Moreover, it was shown

that the procedure was applicable to other plant species including tomato, tobacco, rice and switchgrass with increased duration of co-cultivation (Li *et al.*, 2009).

1.6 Functional assessment of antimicrobial peptides expressed in plants

Function and antimicrobial activity of AMPs depend on three dimensional amphipathic structure of these peptides and their interaction with microbial membranes (Cruz *et al.*, 2014; Lee *et al.*, 2014). Post-translational modifications such as glycosylation, disulphide bond formation and folding are critical for maintaining AMP structure and biological activity. Although these modifications can be performed properly by plant cells, AMPs produced in heterologous plant-based systems might still have slight differences compared to their natural counterparts (Obembe *et al.*, 2011; Viana *et al.*, 2013). Similarly, synthetic AMPs might show different level of activity than that predicted *in silico*, after synthesis in a plant host. Furthermore, translational modifications such as fusions to secretion peptides or subcellular targeting signals, employed to boost expression in plant-based systems might alter the structure, function and stability of AMPs. Hence, evaluation of function and stability of heterologously produced AMPs are critical for intended uses in molecular farming and plant protection.

Standard techniques in molecular biology or plant physiology, such as DNA, RNA and protein blotting, conventional or quantitative PCR, immunological assays, microscopy techniques, mass spectrometry, and many others might be employed to demonstrate the presence of transgene, transcript or the heterologous product in an expression host. These techniques should be accompanied by inhibition tests, bioassays or various other methods to show the activity of the AMP produced in a heterologous system. Functional assessment of AMPs can be performed using *in vivo* or *in vitro* assays depending on the expression system employed, purpose of peptide production, the AMP synthesized and the target pathogen group.

In studies, where improvement of disease resistance in plants was aimed, functional assessment was generally done by challenging transgenic plants with target pathogens. Intact or detached leaves of a transgenic plant expressing an AMP were infiltrated with a suspension of a bacterial pathogen, infected physically with mycelium of a fungus or inoculated mechanically with a viral pathogen (Donini *et al.*, 2005;

Furman *et al.*, 2013; Lee *et al.*, 2011; Muramoto *et al.*, 2012; Yevtushenko and Misra, 2012; Zakharchenko *et al.*, 2013a; 2013b). Whole transgenic tomato plants expressing a cationic peptide (cecropin B) were challenged with pathogens causing bacterial wilt and spot, and were shown to display increased resistance (Jan *et al.*, 2010). Transgenic potato plants over-expressing endogenous Snakin-2 were inoculated with a suspension of *Pectobacterium atrosepticum* at a wounded site on the stem. Transgenic potato lines exhibited enhanced resistance to blackleg disease caused by *P. atrosepticum* according to restrained disease symptoms such as chlorosis, necrosis or stem collapse (Mohan *et al.*, 2014). These bioassays, exemplified here and many others provide valuable information since they present direct evidence for improvement of disease resistance. On the other hand, these assays should include proper controls such as untransformed and mock transformed plants or tissues from these plants.

One of the most widely employed bioassays is inhibition tests performed *in vitro*. Total or crude protein extracts from transgenic plants expressing AMPs are used to inhibit microbial growth in diffusion assays on solid media or in liquid bacterial suspension cultures (Jan *et al.*, 2010; Zakharchenko *et al.*, 2013a; 2013b). Number of viable microbial cells, concentration of AMP in the protein extract, culture conditions, duration of incubation, and various other parameters might affect the results obtained from these inhibition assays. Additionally, antimicrobial activity of contaminating endogenous proteins, peptides or metabolites from the host organism might also compromise the results. Hence, cautiously selected control reactions should be included alongside protein extracts from transgenic plants or cells. Extracts from transgenic tomato expressing cecropin B were added to bacterial liquid suspension cultures containing 1×10^4 colony forming units (CFU) of either *Escherichia coli*, *Salmonella enteritidis*, or *Erwinia carotovora* to evaluate the inhibitory effect of heterologous cecropin B on bacterial growth. According to optical density recordings after incubation for 17 h, growth inhibition of 16 – 35% was determined in bacteria treated with extracts of transgenic tomato compared to wild type plants (Jan *et al.*, 2010). Since there is no single type of peptides, peptide structures or mode of actions, there is no universal bioassay for functional evaluation of AMPs. Instead, various *in vitro* and *in vivo* tests might be developed depending on the nature of plant extracts, a specific AMP and its target and the properties of the expression host.

Regarding the development of method for *in vitro* screening of *in planta* produced recombinant AMPs, consideration should be given to many steps, all of them being critical, as they determine the result of the analysis. These include selection of plant for antimicrobial screening and the tissue analysed, as various plants and their parts might express the target transgene at various level. Attention should be also given to selection of solvent system, time and temperature of extraction. It is crucial to search for extraction methods that gives the highest yields of target antimicrobial chemicals, avoids waste of time and money and does not ineterfere with subsequent antimicrobial activity assay. As compounds presented in the plant crude protein extract may negatively affect analysis, thus give false negative results, further purification of target AMP and its enrichment in a given target volume of tested extract might be employed. Variety of methods for AMPs purification might be used, including the chromatographic separation, where cationic character, hydrophobicity and eventually also presence of various purification tags are taken into advantage. When workinng with AMP fusion product, a special emphasis should be given to effective removal of the tag (cleavage buffer, temperature, time etc.). After final preparation of the tested and control sample, selection of target microorganism for antimicrobial susceptibility testing, the size of inoculum, its quantification and preparation, type of antimicrobial screening method, time and temperaure of incubation and type of growth medium may influence the results of analysis. As there is no report describing *in vitro* functional assessment of plant produced LL-37 or its analogues, all of the aforementioned parameters had to be optimized in frame of this thesis to avoid overlooking of biological activity of LL-37 peptide.

2 Materials and methods

2.1 Transient expression of chimeric *LL-37* genes in *tobacco*

To select the most desirable plant-based production strategy of recombinant LL-37 (rLL-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 (Peroutka *et al.*, 2008) 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 (E) or the Factor Xa (X) recognition site were 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 synthesised by Thermo Fisher Scientific, USA. All DNA coding sequences were inserted using T4 DNA ligase into pENTR 2B Dual Selection Vector (Thermo Fisher Scientific, USA) through *Sal*I and *Eco*RI restriction sites creating intermediate plasmids. Potentially positive *E.coli* TOP10 cells were selected using Luria-Bertani (LB) plates supplemented with either kanamycin (75 μg/ml) or chloramphenicol (12.5 μg/ml), respectively. The genes were then subcloned into the *Xho*I enzyme linearized binary vector pGWB17 (Fig. 6A) downstream of the CaMV 35S promoter (35Sp) via Gateway LR recombination (Thermo Fisher Scientific). The next step included transformation of created plasmids into *E. coli* TOP10 cells and selection of bacterial clones on LB media supplemented with kanamycin (75 μg/ml) plus hygromycin (50 μg/ml).

Prepared constructs of recombinant human LL-37 (rLL-37) included:

pGWB17/35Sp::ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL::Nos-t;
pGWB17/35Sp::ZmCKX1sp_6xHis_MBP_E_LL-37::Nos-t;
pGWB17/35Sp::6xHis_MBP_E_LL-37::Nos-t;
pGWB17/35Sp::ZmCKX1sp_6xHis_SUMOstar_LL-37::Nos-t;
pGWB17/35Sp::6xHis_SUMOstar_LL-37::Nos-t;
pGWB17/35Sp::ZmCKX1sp_6xHis_pHvSUMO_E_LL-37::Nos-t;
pGWB17/35Sp::6xHis_pHvSUMO_X_LL-37::Nos-t;
pGWB17/35Sp::ZmCKX1sp_LL-37_KDEL::Nos-t;
pGWB17/35Sp::ZmCKX1sp_LL-37::Nos-t;
pGWB17/35Sp::LL-37::Nos-t.

Their overall scheme is shown in Fig. 6B and the corresponding amino acid sequences are listed in Supporting Information, Fig. S1A-J. As a final step, all of the obtained plant expression vectors were inserted via electro-transformation into *A. tumefaciens* strain GV3101, that was spread over MG/L media supplemented with following antibiotics: rifampicin (10 μg/ml), gentamycin (50 μg/ml), kanamycin (100 μg/ml) and hygromycin (50 μg/ml). The presence of the entire cloning cassettes was verified by back-transformation of plasmids isolated from *A. tumfeaciens* to the E. coli TOP 10 cells and subsequent sequencing by a commercial service (SEQme, Czech Republic).

Tobacco plants (3 weeks old, *Nicotiana benthamiana* L.) were agroinfiltrated according to a published protocol (Sparkes *et al.*, 2006). 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) was injected into tobacco tissue. Infiltrated area of approximately 3 to 5 cm^2 was collected and pooled from all leaves to analyse the expression of *LL-37* chimeric genes on both RNA and protein level.

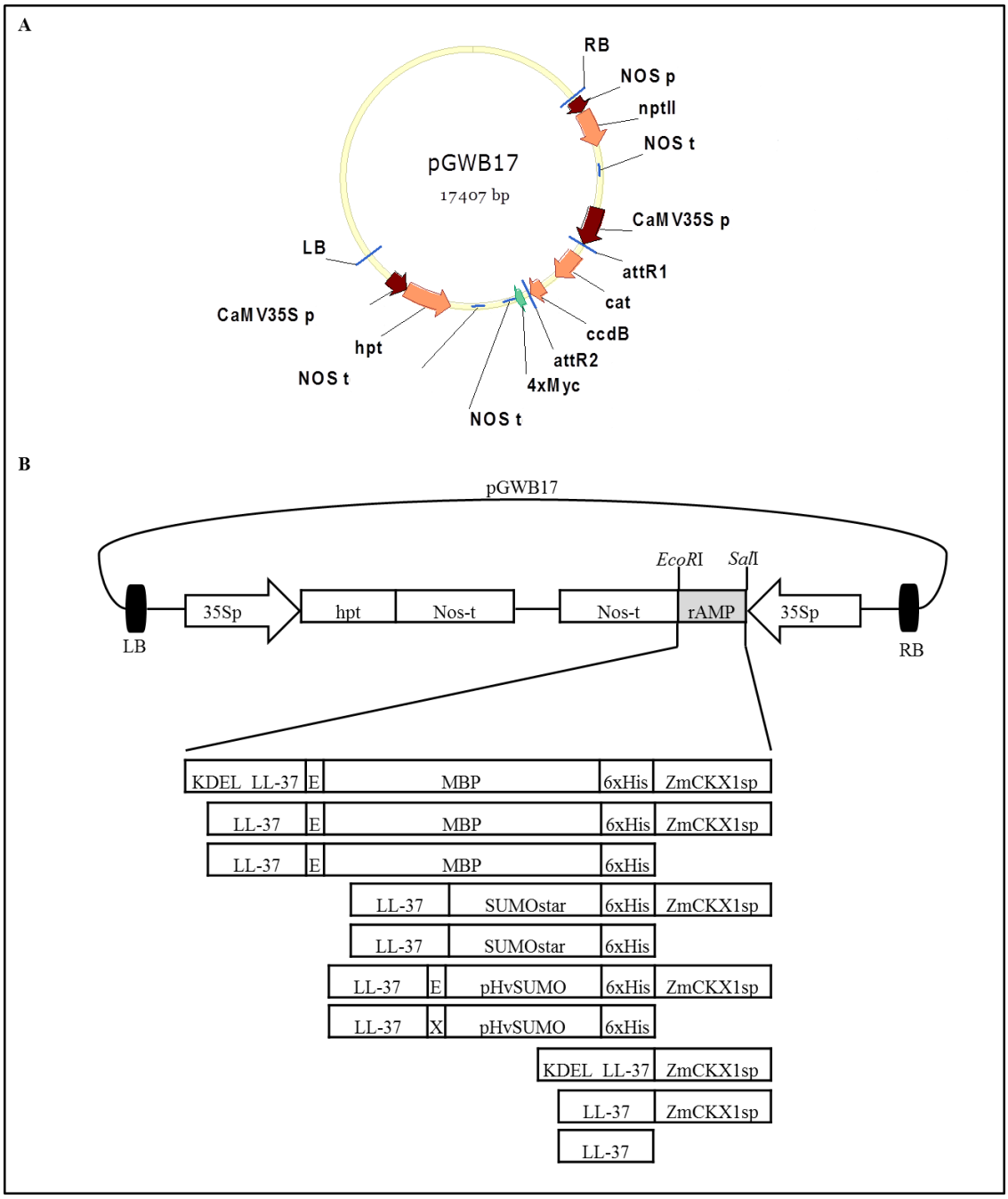

Figure 6. Map of the expression vector pGWB17 with orientation of individual functional segments marked by arrows (A) and schematic diagrams of the gene constructs used for an ectopic expression of recombinant human LL-37 in tobacco leaf tissue (B). Cloning sites are indicated. LB, left border; RB, right border; 35Sp, 35S promoter from the cauliflower mosaic virus; hpt, hygromycin resistance selectable marker gene; Nos-t, the terminator of nopaline synthase gene of *Agrobacterium tumefaciens*; rAMP, recombinant antimicrobial peptide; LL-37, human cathelicidin antimicrobial peptide; ZmCKX1sp, *Zea mays* cytokinin oxidase/dehydrogenase 1 signal peptide; KDEL, endoplasmic reticulum retention signal peptide; 6xHis, polyhistidine affinity 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.

2.2 qPCR analysis for selection of barley endosperm specific promoter suitable for expression of antimicrobial peptides

Barley native genes that are specifically expressed in endosperm were identified using Genevestigator Affymetrix 22K Barley GeneChip array (Hruz *et al.*, 2008). The strength and temporal profile of candidate promoters was analysed 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 (Table 4), which were designed with *Primer Express* Software 3.0.1 (Thermo Fisher Scientific). The expression profile was analysed in six different phenological growth stages of barley on BBCH scale (Lancashire *et al.*, 1991) i.e. 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). 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 at least 2 independent biological samples and each sample was run in 3 technical replicates. In order to further investigate tissue specificity of B1 hordein gene promoter (*B-HORp*), seeds of barley T2 homozygous plants overexpressing *HvCKX9* gene (barley cytokinin oxidase/dehydrogenase 9 gene) under the control of *B-HORp* and Nos-t (plants transformed with *pBRACT209/HORp::HvCKX9::Nos-t* expression casette) were analysed using qPCR. These plants were prepared in the scope of my master thesis (Holásková, 2012). For the purpose of qPCR, aliquots of 1.5 ng of cDNA isolated from endosperm, embryo, aleurone and seed coat tissues of late milk grains (GS77) were mixed with SYBR® Green Power PCR Mix and forward and reverse primer of final concentration 300 nM. Next, the expression of *HvCKX9* was analysed on ViiA7 Real-Time PCR System using the mRNA levels of barley transcriptional elongation factor 2 (*HvEF2*) and cyclophilin (*HvCYC*) genes as quantitative controls. Reaction conditions were set up as already described above in this subchapter. Each experiment was set up for 3 independent lines and each line was analysed in 3 biological and 3 technical replicates. The specificity of the amplification was proved by an analysis of melting curves. The messenger RNA (mRNA) levels of barley transcriptional elongation factor 2 (*HvEF2*) and cyclophilin (*HvC*) genes served as

quantitative controls. Expression data were analysed using the DataAssist software (Thermo Fisher Scientific).

Table 4: Oligonucleotides used to select barley native endosperm specific promoter by qPCR analysis

Primer	Sequence 5'→3'	Amplified region
B-Hor_F [S]	TTGCAGCCACACCAGATAGC	B1 hordein gene
B-Hor_R [S]	GGTACGCAGCGCAATGG	B1 hordein gene
D-Hor_F [S]	CCTCTTTGTGGCGGTAATCG	D hordein gene
D-Hor_R [S]	TTCCCATTGATCTCACGTTCAG	D hordein gene
Hinb_F [S]	ACAAATTCAGAGGGCCCAAA	hordoindoline b
Hinb_R [S]	TACAGTCGGCGCCCATGT	hordoindoline b
CI2_F [S]	AAGCCGGAGGGAGTGAACA	chymotrypsin inhibitor 2
CI2_R [S]	GGCCACTCTGTCTTCTGGTTTT	chymotrypsin inhibitor 2
TI_F [S]	GGATGCGTTGCCACACAA	trypsin inhibitor
TI_R [S]	GGCAGATTTGACTGACCACGTA	trypsin inhibitor
HvEF2_F [S]	CCGCACTGTCATGAGCAAGT	transcriptional elongation factor 2
HvEF2_R [S]	GGGCGAGCTTCCATGTAAAG	transcriptional elongation factor 2
HvC_F [S]	CCCAGTTCTACATAACCACAATCAA	cyclophilin
HvC_R [S]	ACCCTGCCAAAGACTACATGCT	cyclophilin
HvCKX9_F [S]	TGGAGCAATATGTCTATGTTAGTATGGA	CKX/CKO 9
HvCKX9_R [S]	TCTTCACGCTGCAGTTCGTT	CKX/CKO 9

"F" denotes forward, and "R" denotes reverse primer orientation
"S" denotes SYBR® Green chemistry
CKX/CKO 9 denotes cytokinin oxidase/dehydrogenase 9

2.3 Analysis of temporal activity of B1 hordein gene promoter

The B-HORp activity dynamics was analysed in T2 generation of homozygous transgenic barley plants with integrated pBRACT209/HORp::HvCKX9::Nos-t expression casette (see the chapter 2.2) using the cytokinin oxidase/dehydrogenase assay (Frébort *et al.*, 2002). First, spikes of 6 different growth stages (GS41, GS49, GS59, GS77, GS83, and GS87) were frozen in liquid nitrogen and homogenized using mortar and pestle. Subsequently, an extraction buffer (200 mM Tris/HCl pH 8.0 containing 1 mM phenylmethylsulfonylfluoride and 0.3% Triton X-100) was added to the samples in the ratio of 1:1.5 (w/v) and the samples were incubated on ice for 60 min. The cell debris was then removed by centrifugation at 18 000 x*g* at 4 °C for 10 min. Supernatants were transferred to fresh tubes and used for determinig the protein content using Bradford assay with a bovine serum albumin as standard (Bradford, 1976) and specific CKX/CKO activity by the endpoint 4-aminophenol assay (Frébort et al., 2002).

The *in vitro* CKX/CKO activity was estimated by a spectrophotometric assay, which was performed as follows: extracted proteins (0.1-0.8 mg depending on the developmental stage analysed) were mixed with 0.2 mM N^6-isopentenyladenine-9-glucoside (iP9G) as a substrate and 0.5 mM potassium ferricyanide as artificial electron acceptor in McIlvaine buffer (pH 5.0). Total reaction volume was 590 µl. After overnight incubation at 37 °C, the reaction was stopped by an addition of 300 µl 40% (w/v) UBItrichloroacetic acid (TCA) together with 200 µl of 2% 4-aminophenol in 6% TCA. After incubation at 20 °C for 5 min followed by a centrifugation at 18 000 x*g* at 4 °C for another 5 min the absorbance at 352 nm was measured against blank that excluded the substrate (Frébort et al., 2002).

2.4 Construction of binary vectors for expression of chimeric *LL-37* genes in transgenic 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 (*B-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 *B-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 Centre, Norwich, UK; Fig. 7A) 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; Fig. 7B) through LR Gateway® reaction downstream of *UBIp*. The aforementioned cloning methods resulted in constructs:

pBRACT209/B-HORp::ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL::Nos-t;
pBRACT209/B-HORp::ZmCKX1sp_LL-37_KDEL::Nos-t;
pBRACT209/B-HORp::ZmCKX1sp_LL-37::Nos-t;
pBRACT209/B-HORp::ZmCKX1sp_$(GGGGS)_2$_6xHis_$(GGGGS)_2$_E_LL-37::Nos-t;
pBRACT209/B-HORp::OsCht11sp_6xHis_E_LL-37::Nos-t;
pBRACT214/UBIp::ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL::Nos-t;
pBRACT214/UBIp::ZmCKX1sp_LL-37_KDEL::Nos-t;
pBRACT214/UBIp::ZmCKX1sp_$(GGGGS)_2$_6xHis_$(GGGGS)_2$_E_LL-37::Nos-t;

Fig. 7C and 7D illustrate the scheme of the final constructs used in this thesis. Sequences of all of the binary plasmids (see Supporting Information, Fig. S2A-G) were inserted into chemically competent *E.coli* TOP10 cells that were plated over LB agar plates supplemented with kanamycin (75 μg/ml) or chloramphenicol (12.5 μg/ml) for positive and negative selection, respectively (New England Biolabs, USA). After final verification of identity of the plasmids using conventional techniques (PCR, Table 5; restriction enzyme digest), were all of the expression vectors inserted via electroporation into *Agrobacterium tumefaciens* strain AGL1 (obtained from Plant Breeding and Acclimatization Institute, Blonie, Poland) together with the helper plasmid pSoup and the identity of individual cloning cassettes was confirmed

by commerctial sequencing (SEQme, Czech Republic) of back-transformated plasmids from *A. tumefaciens* to the *E. coli* TOP 10 cells.

Table 5: Oligonucleotides used for confirming of rLL-37 variants in pBRACT209 and pBRACT214 by PCR

Primer	Sequence 5'→3'	Amplified region	Amplicon size (bp)
hpt_F	CGAAAAGTTCGACAGCGTC	hygromycin phosphotransferase gene	649
hpt_R	GGTGTCGTCCATCACAGTTTG	hygromycin phosphotransferase gene	649
B-HORp_R	TCCATTCTTGTTTCAGGCTAAC	barley B1 hordein promoter	*
LL-37_F	GCCGATCTTCTCCTTGGACTT	human cathelicidin LL-37 gene	*
hpt_nos_F	ACCGATGGCTGTGTAGAAGTAC	hygromycin phosphotransferase gene	*
pBRACT214_F	CCCTGCCTTCATACGCTATT	*UBIp*, attR1, chloramphenicol resistance gene, *ccdb* gene, attR2, *Nos-t*	*
pBRACT214_R	TGTTTGAACGATCCTGCTTG	*UBIp*, attR1, chloramphenicol resistance gene, *ccdb* gene, attR2, *Nos-t*	*

"F" denotes forward, and "R" denotes reverse primer orientation
"*" means that amplicon sizes depend on type of the expression vector analysed

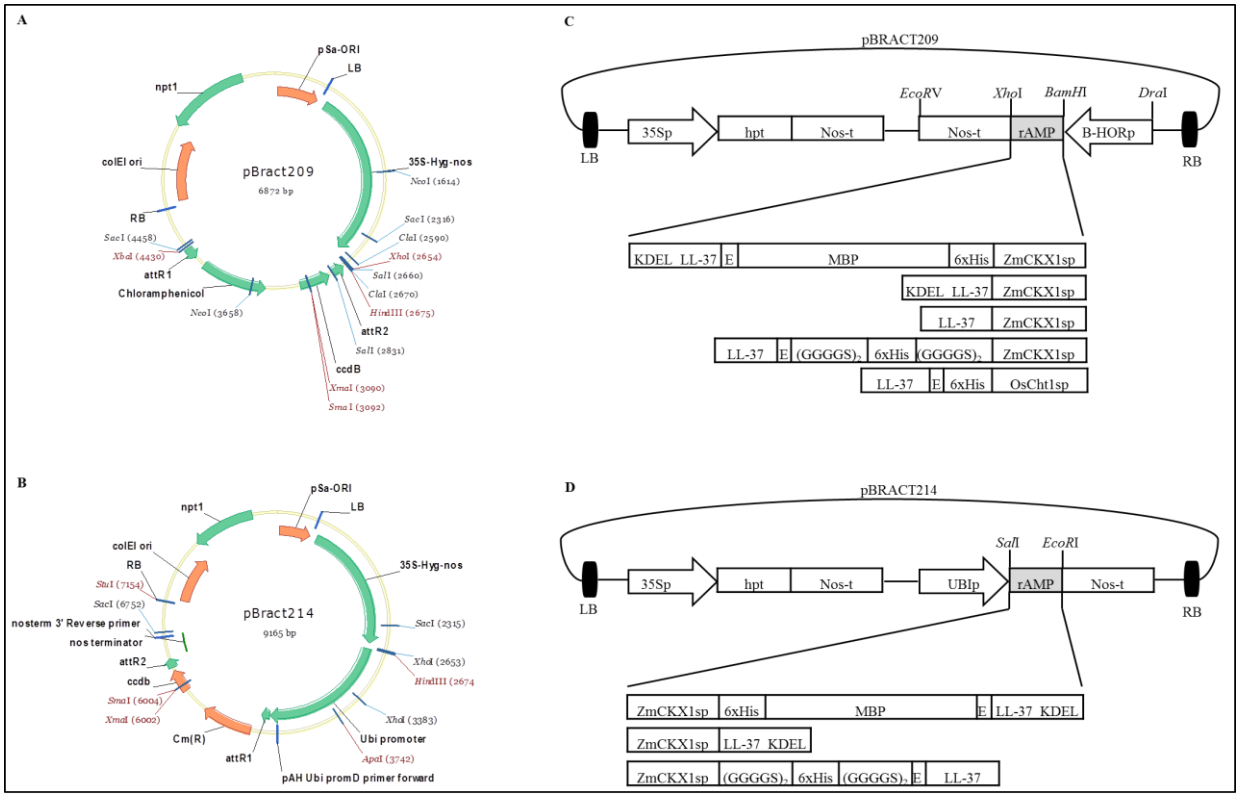

Figure 7. Maps of the expression vectors of the pBRACT series with the orientation of individual functional segments marked by arrows (A, B) and schematic diagrams of the gene constructs used for ectopic expression of recombinant human LL-37 in barley (C, D). Cloning sites are indicated. LB, left border; RB, right border; 35Sp, 35S promoter from the cauliflower mosaic virus; B-HORp, barley B1 hordein gene promoter; UBIp, maize ubiquitin gene promoter; hpt, hygromycin resistance selectable marker gene; Nos-t, the terminator of nopaline synthase gene 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 reticulum retention signal peptide; 6xHis, polyhistidine affinity tag; MBP, maltose binding protein tag; E, enterokinase recognition site; X, Factor Xa recognition site; (GGGGS)2, flexible peptide linker.

2.5 Construction of binary vectors for expression of chimeric *HBD2* and *PEX* genes in transgenic barley

Plasmid expression vector for grain specific production of the recombinant pexiganan (rPEX) antimicrobial peptide in trasgenic barley lines consisted of DNA sequence coding for the antimicrobial peptide attached to C-terminal KDEL tetrapeptide. The chimeric antimicrobial peptide coding region was bordered by *BsiW*I and *EcoR*I restriction enzyme cleavage sites on its N- and by *Xho*I and *Avr*II multiple cloning sites on its C- termini. The expression of chimeric pexiganan gene (*rPEX*) was placed under the control of the barley endosperm-specific trypsin inhibitor promoter (*TIp*, GenBank: X65875.1) and the terminator of nopaline synthase gene (Nos-t). A sequence encoding recombinant human beta-defensin 2 (rHBD2) was supplemented at the N-terminus with the sequence coding for 8x His tag. Furthermore, the enterokinase recognition site encoding sequence was placed between the *HBD2* and 8x His tag. The resulting sequence was extended with sequences of *BsiW*I and *EcoR*I cleavage sites on its 5' end, and *Xho*I and *Avr*II DNA cleavage sites on its 3' end. Expression of *rHBD2* was driven by the endosperm-specific oat globulin promoter (*AsGlo1p*, GenBank: AY795082.1) and the Nos-t. Codon usage of the *PEX* and *HBD2* chimeric genes was optimized for the expression in barley. Finally, to enhance the initiation of translation, the monocot Kozak sequence was introduced into both of the synthetic DNA molecules. In addition, both of the genes were flanked by *att*L recombination sites to allow the gateway recombination cloning. Resulting constructs were commercially synthesized by Thermo Fisher Scientific and digested by *Bgl*II (for *PEX*) and *Nco*I (for *HBD2*) enzymes to obtain linearized plasmids. The individual sequences were then inserted into pBRACT209 destination vectors (www.bract.org, provided by John Innes Centre, Norwich, UK, Fig. 7) by Gateway® LR recombination cloning (Thermo Fisher Scientific) using Gateway™ LR Clonase™ II Enzyme mix. This generated the pBRACT209/TIp::PEX_KDEL::Nos-t and the pBRACT209/AsGLO1p::8xHis_E_HBD2::Nos-t expression vector. The overall scheme of the prepared vectors is shown in Figure 8A and 8B. Sequences of the resulting constructs (see Supporting Information, Fig. S2H,I) were then inserted into chemically competent *E. coli* TOP10 cells that were spread on LB agar plates supplemented with following antibiotics (New England Biolabs, USA): kanamycin (75 μg/ml) for positive selection, or chloramphenicol (12.5 μg/ml) for negative selection. After selection of potentially positive clones, the identity of the final

constructs was verified by colony PCR and restriction analysis. Final constructs were introduced into the hypervirulent *Agrobacterium tumefaciens* strain AGL1 (obtained from Plant Breeding and Aclimatization Institute, Blonie, Poland) by electroporation and finally commercialy sequenced by SEQme.

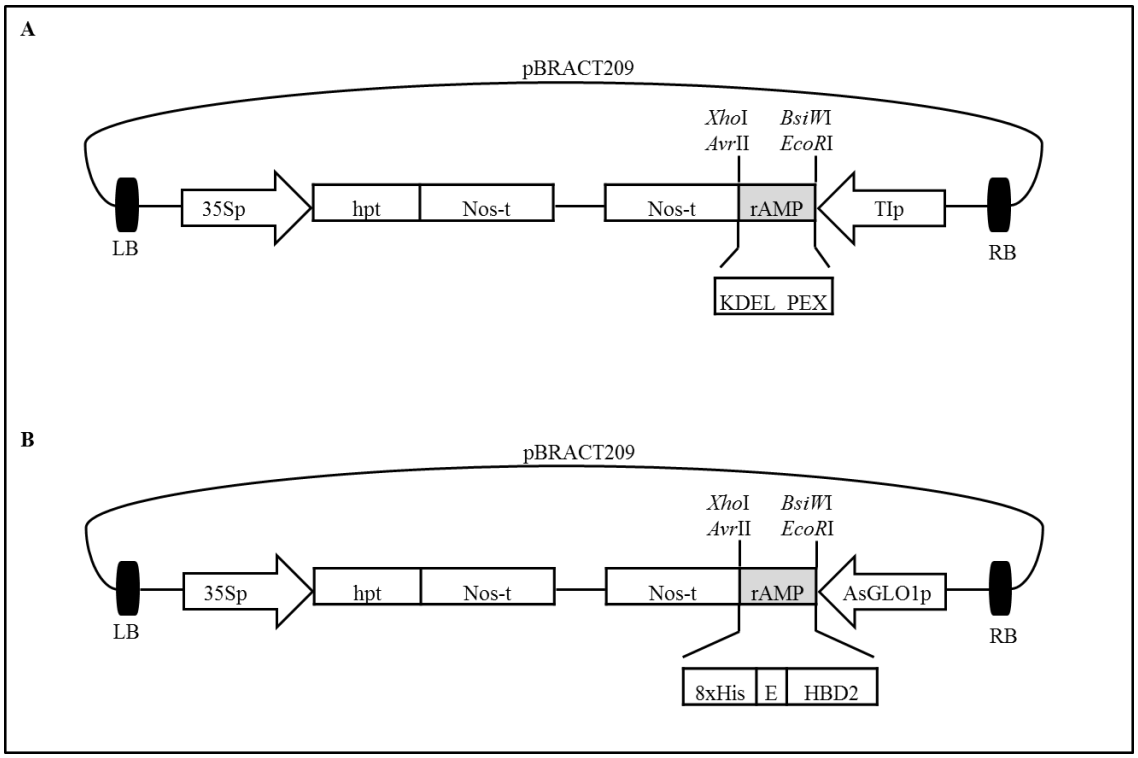

Figure 8. Schematic diagrams of the gene constructs used for ectopic expression of recombinant peptides pexiganan (A) and human beta-defensin 2 (B) in barley. Cloning sites are indicated. LB, left border; RB, right border; 35Sp, 35S promoter from the cauliflower mosaic virus; TIp, barley trypsin inhibitor gene promoter; AsGLO1p, oat globulin gene promoter; hpt, hygromycin resistance selectable marker gene; Nos-t, the terminator of nopaline synthase gene of *Agrobacterium tumefaciens*; rAMP, recombinant antimicrobial peptide; PEX, pexiganan; HBD2, derivative of human beta-defensin 2; KDEL, endoplasmic reticulum retention signal peptide; 8xHis, polyhistidine affinity tag; E, enterokinase recognition site.

2.6 Genetic transformation and selection of transgenic barley plants expressing antimicrobial peptides

For stable transformation of spring barley (*Hordeum vulgare* L. cv. Golden Promise), the hypervirulent *A. tumefaciens* AGL1 strain was used to generate transgenic lines carrying always one of the abovementioned binary vectors (Fig. 7C, 7D, 8A, 8B). Genetic modification of wounded immature barley embryos was performed basically

according to a previously described protocol (Harwood *et al.*, 2009) 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 of potentially transgenic as well as control plants were transferred into hydrated peat jiffy pellets (Rosteto, Czech Republic) and grown under following growth conditions: 15 °C/16 h/light (140 μM/m^2/s) and 12 °C/8 h/dark cycles at 60% of relative humidity. 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 eventually also 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 (CNT).

2.7 Screening of regenerated barley plants for transgene integration via PCR amplification of genomic DNA

To analyse transgene presence and its inheritance, plant genomic DNA was extracted from leaves of approximately four week old plants, essentially as described previously (Pallotta *et al.*, 2000). Plants were screened for the presence of both the *hpt* gene fragment as well as the *promotor::AMP* gene fusion using primers listed in Table 6. PCR reaction consisted of initial denaturation at 95 °C for 2 min, followed by a series of 35 cycles of thermocycling under these conditions: 95 °C for 30 s, 55 °C for 30/or 102 s (for *hpt, TIp::PEX, AsGLO1p::HBD2/*or *promoter::rLL-37* detection, respectively), 72 °C for 30 s. Final elongation was performed at 72 °C for 10 min. 12.5 μl aliquots of reaction mixture contained 50-100 ng of gDNA, 1x Taq flexi reaction buffer, 1.5 mM $MgCl_2$, 0.2 μM forward primer, 0.2 μM reverse primer, 0.2 μM dNTPs, and 0.35 U of GoTaq polymerase. Only plants with both of the amplification products of expected sizes, thus most likely possessing complete expression cassette, were further analysed and eventually propagated to next generations.

Table 6: Oligonucleotides used for genotypization of barley plants by PCR

Primer	Sequence 5'→3'	Amplified region	Amplicon size (bp)
hpt_F	CGAAAAGTTCGACAGCGTC	hygromycin phosphotransferase gene	649
hpt_R	GGTGTCGTCCATCACAGTTTG	hygromycin phosphotransferase gene	649
B-HORp_R	TCCATTCTTGTTTCAGGCTAAC	barley B1 hordein promoter	*
LL-37_F	GCCGATCTTCTCCTTGGACTT	human cathelicidin LL-37 gene	*
UBIp_F	TGCTCACCCTGTTGTTTGGTGTTAC	maize ubiquitin promoter	*
TIp_F	CCAGCTGCTTGTTCACTTCACA	barley trypsin inhibitor promoter	173
PEX_R	CTTCTTCAGGATCTTCACGAAGG	pexiganan gene	173
AsGLO1p_F	AGGAGTCACAAGTGCCACAAAC	oat globulin promoter	482
HBD2_R	CACGTGCCGATCTGCTTGT	human beta-defensin 2 gene	482

"F" denotes forward, and "R" denotes reverse primer orientation
"*" means that amplicon sizes depend on type of the expression vector used for the transformation

2.8 Estimation of transgene copy number based on Southern blot analysis

Some of the PCR-positive plants were selected for Southern blot analysis to determine the number of T-DNA inserts, and thus to further define the genome of transgenic barley. For this purpose, 40 μg of genomic DNA (isolated according to Pallota *et al.*, 2000) was digested with *Xho*I, obtained fragments separated by electrophoresis in 0.8% agarose gel and transferred onto a blotting nylon membrane (type B, Merck, Germany). For detection of the inserts, *hpt* gene-specific digoxigenin (DIG)-labelled DNA hybridization probe (for primers see Table 7) was synthesized

using a commercial kit (Roche, Switzerland). A reaction mixture of 25 µl consisted of 200 pg of template DNA (empty pBRACT207 plasmid), 1x PCR buffer with $MgCl_2$, lx PCR DIG Labeling Mix, 1x dNTP stock solution, 1 µM forward primer, 1 µM reverse primer, 1.33 U of enzyme mix, and nuclease-free water. A reaction mixture for synthesis of unlabeled positive control was identical to the one described above, but without the 1x PCR DIG Labeling Mix. Amplification conditions of the PCR reaction were set up as follows: denaturation at 95 °C for 2 min, after that series of 10 cycles of thermocycling at 95 °C for 10 s, 60 °C for 30 s and 72 °C for 2 min. That was followed by another series of 30 cycles of thermal cycling at 95 °C for 10 s, 60 °C for 30 s and 72 °C for 2 min extended with additional 20 s for each successive cycle. Final elongation was performed at 72 °C for 7 min. After that were membranes exposed to *hpt* gene-specific DIG-labelled DNA hybridization probe 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. There were few steps during this analysis that had to be carefully planned and handled to get results of high quality. These included: working with relatively fresh gDNA samples (either with fresh isolates or with those being not stored at -80 °C for more than few months); complete digestion of the right quantity of gDNA; careful handling of the membranes to avoid unspecific background and perfoming of prehybridization as well as hybridization at 46 °C.

Table 7: Oligonucleotides used for DNA probe synthesis

Primer	Sequence 5'→3'	Amplified region	Amplicon size (bp)
hpt_probe_F	GAATTCAGCGAGAGCCTGAC	hygromycin phosphotransferase gene	557
hpt_probe_R	ACATTGTTGGAGCCGAAATC	hygromycin phosphotransferase gene	557

"F" denotes forward, and "R" denotes reverse primer orientation

2.9 Estimation of transgene copy number based on a segregation analysis

Transgene copy number was determined according to PCR detection of hygromycin (*hpt*) gene. For this purpose, all seeds from a single mature spike of each analysed T0 plant were put to the soil to produce T1 plants, which were subsequently screened by PCR (see the chapter 2.7) for the presence of *hpt* transgene to determine the Mendelian ratio.

2.10 Flow cytometric detrmination of DNA ploidy level of primary barley transformants

The ploidy level of primary transformants was determined by flow-cytometry using ML CyFlow flow cytometer (Partec, Germany) at an early stage of development (Doležel *et al.*, 2007). Leaf tissue was chopped in LB01 isolation buffer containing 2% w/v polyvinylpyrrolidone to prevent the interference of phenolic compounds with DNA staining (Doležel and Bartoš, 2005). The acquired suspension of nuclei was stained with 0.01% w/v 4′,6-diamidino-2-phenylindole (DAPI). Leaves of non-transformed barley were used as internal reference standard.

2.11 Selection of homozygous transgenic barley lines

Homozygous lines were selected from diploid T1 transgenic barley lines possessing either 1 or 2 copies of the insert (based on results from Southern blot or segregation analysis). All the seeds from a single spike of each of the analysed plants were sterilized, and their embryos were isolated under sterile conditions and put on regeneration media (2.7 g/l Murashige and Skoog modified plant salt base without NH_4NO_3 (M0238, Duchefa, The Netherlands), 20 g/l maltose, 165 mg/l NH_4NO_3, 750 mg/l glutamine, 100 mg/l myo-inositol, 0.4 mg/l thiamine HCl, 3.5 g/l Phytagel, pH 5.8) with hygromycin for selection (50 mg/ml). The embryos were incubated in environmental chamber under the selection conditions (the chapter 2.6) for 2 weeks. Embryos originated from non transgenic plants were used as a negative control. Only those T1 lines whose all embryos gave rise a T2 plantlets under the used selection pressure were further analysed. The homozygozity was finally proven by screening for the presence of the *hpt* transgen as already described in the chapter 2.7.

2.12 Analysis of *rAMPs* gene expression

RNA was extracted from tobacco leaves and from barley roots, leaves and grains that included BBCH 73, 85 and 87 in case of *PEX* and *HBD2* expressing lines, and late milk endosperm grains (BBCH 77) in case of *LL-37* expressing lines. For isolation of total RNA and subsequent removal of residual contaminating DNA, RNAqueous kit and Turbo DNase (Thermo Fisher Scientific) were used, respectively. Further purification of the samples was performed with Agencourt RNAClean XP (Beckman Coulter, USA). cDNA synthesis was accomplished from 2 µg of purified total RNA using the M-MuLV RT reverse transcriptase (Thermo Fisher Scientific). *rLL-37* gene expression was assessed by reverse transcription polymerase chain reaction 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 analysed on 3% agarose, stained with ethidium bromide and visualised 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. Furtherome, some of the transgenes expressed in tobacco were subjected to qPCR analysis on StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific) using SYBR Green reaction with gene specific oligonucleotides (Table 8), which were designed with *Primer Express* Software 3.0.1 (Thermo Fisher Scientific). Conditions of qPCR reaction were set up as already described in the chapter 2.2. All corresponding primer sequences are listed in Table 8.

Table 8: Oligonucleotides used for (q)RT-PCR gene expression analysis

Primer	Sequence 5'→3'	Amplified region	Amplicon size (bp)	Analysed plant	Analysed variant of LL-37 gene
HvACT_F	TGTTGACCTCCAAAGGAAGCTATT	barley actin gene	73	B	
HvACT_R	GGTGCAAGACCTGCTGTTGA	barley actin gene	73	B	
HvEF2_F	AAGTCCTGCCGCACTGTCAT	barley elongation factor 2 gene	60	B	
HvEF2_R	GGGCGAGCTTCCATGTAAAG	barley elongation factor 2 gene	60	B	
NbeACT_F	GCCCTGAGGTCCTTTTCCA	tobacco actin gene	135	T	
NbeACT_R	CCACCACTGAGCACTATGTTTCC	tobacco actin gene	135	T	
NbeEF1_F	TCCCCATCTCTGGTTTCGA	tobacco elongation factor 1 gene	114	T	
NbeEF1_R	GGCCTCTTGGGCTCATTAATC	tobacco elongation factor 1 gene	114	T	
8_F	GGATCCGCCACCATGCT	cathelicidin LL-37 gene	66	B, T	LL-37

8_R	TGAACTCCTTGCCGATCTTCTC	cathelicidin LL-37 gene	66	B, T	LL-37
9+10_F	GCCTCATTGCCTGCTCTCA	cathelicidin LL-37 gene	64	B, T	ZmCKX1sp_LL-37, ZmCKX1sp_LL-37_KDEL
9+10_R	GCCGATCTTCTCCTTGGACTT	cathelicidin LL-37 gene	64	B, T	ZmCKX1sp_LL-37, ZmCKX1sp_LL-37_KDEL
MBP_F	AGGCCCTCTCCCTGATCTACA	maltose binding protein gene	134	B, T	ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL, ZmCKX1sp_6xHis_MBP_E_LL-37, 6xHis_MBP_E_LL-37
MBP_R	AGTACGGCTCCTGGAGGTTGA	maltose binding protein gene	134	B, T	ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL, ZmCKX1sp_6xHis_MBP_E_LL-37, 6xHis_MBP_E_LL-37
11_F	AGCCACCATCACCATCATCA	cathelicidin LL-37 gene	117	B	ZmCKX1sp_$(GGGGS)_2$_6xHis_$(GGGGS)_2$_E_LL-37
11_R	TGAACTCCTTGCCGATCTTCTC	cathelicidin LL-37 gene	117	B	ZmCKX1sp_$(GGGGS)_2$_6xHis_$(GGGGS)_2$_E_LL-37
12_F	GGCCACCATCACCATCATC	cathelicidin LL-37 gene	79	B	OsCht11sp_6xHis_E_LL-37
12_R	TTGCCGATCTTCTCCTTGGA	cathelicidin LL-37 gene	79	B	OsCht11sp_6xHis_E_LL-37
SUMOstar_F	TCCTCTACGACGGCATCGA	SUMOstar gene	71	T	ZmCKX1sp_6xHis_SUMOstar_LL-37, 6xHis_SUMOstar_LL-37
SUMOstar_R	TGATGTCGTTGTCCTCCATGTC	SUMOstar gene	71	T	ZmCKX1sp_6xHis_SUMOstar_LL-37, 6xHis_SUMOstar_LL-37
pHvSUMO_F	AGGTCCACCCAGCTCAAGAA	putative barley SUMO gene	78	T	ZmCKX1sp_6xHis_pHvSUMO_E_LL-37, 6xHis_pHvSUMO_X_LL-37
pHvSUMO_R	ACAGGAAGGCGATCGAGTTG	putative barley SUMO gene	78	T	ZmCKX1sp_6xHis_pHvSUMO_E_LL-37, 6xHis_pHvSUMO_X_LL-37

"F" denotes forward, and "R" denotes reverse primer orientation

"B" and "T" means that analysed plant material originated from barley and tobacco, respectively

Expression of transgenic RNA of *PEX* expressing lines was relatively quantified on ViiA7 Real-Time PCR System using SYBR® chemistry, and expression of *HBD2* was analysed on StepOnePlus™ system using TaqMan® chemistry. For analysis of *PEX* expressing lines, 10 µl of qPCR reaction mixture contained gb SG PCR Master Mix (Generi Biotech, CZ), 100 nM ROX passive reference dye, 1.5 ng of cDNA, 300 nM forward primer and 300 nM reverse primer. 10 µl of reaction mixture for analysis of *HBD2* expressing lines was composed of gb Ideal PCR Master Mix (Generi Biotech, CZ), 500 nM ROX, 1.5 ng of cDNA, 300 nM forward primer and 300 nM reverse primer, and 250 nM gene specific qPCR probe featuring a 5´6-carboxyfluorescein (FAM) dye and 3´end 5(6)-carboxytetramethylrhodamine (TAMRA) quencher. Furthermore, to see wheter there is some correlation between transgene copy number and transgene expression level, cDNA from grains (BBCH 77) of *ZmCKX1sp_(GGGGS)$_2$_6xHis_(GGGGS)$_2$_E_LL-37* expressing lines was analysed by qPCR to relatively compare the level of transgenic RNA across individual plants. The analysis was performed on StepOnePlus™ using TaqMan® chemistry. 10 µl of reaction mixture was composed of gb Ideal PCR Master Mix, 500 nM ROX passive reference dye, 1.5 ng of cDNA, 300 nM forward primer and 300 nM reverse primer, and 250 nM gene specific qPCR probe featuring a 5´ FAM and a 3´end TAMRA. qPCR analysis was carried out using default thermal cycling conditions (Life Technologies). The mRNA levels of barley β-actin (*HvACT*), transcriptional elongation factor 2 (*HvEF2*) and cyclophilin (*HvCYC*) genes were used as quantitative control. All primers were designed using the Primer Express 3.0 software and their sequences are listed in Table 9.

Table 9: Oligonucleotides used for qPCR analysis of expression

Primer	Amplified region	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')	Probe sequence (5'→3')
HvEF2 [T]	barley elongation factor 2	AAGTCCTGCCGCACTGTCAT	GGGCGAGCTTCCATGTAAAG	AGCAAGTCCCCCAACAAGCATAACCG
HvACT [T]	barley β-actin	TGTTGACCTCCAAAGGAAGCTATT	GGTGCAAGACCTGCTGTTGA	TGTAGTATTCAGCTGGTTGGTGGCACAGC
HvC [T]	barley cyclophilin	TGTCTATGGATTTGACACCACTCTTT	GAAGCCTGCCCGAAGCA	TGACCTGTTTTCTTCGCACACCAGCC
HBD2 [T]	human beta-defensin 2	AAGTCTGGCGCCATTTGC	CACGTGCCGATCTGCTTGTA	ACCCAGTGTTCTGCCCAAGGCG
11 [T]	cathelicidin LL-37 gene	AGCCACCATCACCATCATCA	TGAACTCCTTGCCGATCTTCTC	CGGCTCAGATGACGACGACAAGCTC
HvEF2 [S]	transcriptional elongation factor 2	CCGCACTGTCATGAGCAAGT	GGGCGAGCTTCCATGTAAAG	-
HvACT [S]	barley β-actin	TTGACCTCCAAAGGAAGCTATTCT	GGTGCAAGACCTGCTGTTGA	-
HvCYC [S]	barley cyclophilin	CCCAGTTCTACATAACCACAATCAA	ACCCTGCCAAAGACTACATGCT	-
PEX [S]	pexiganan	AAGTTCCTCAAGAAGGCCAAGAA	CTTCTTCAGGATCTTCACGAAGG	-

"T" denotes TaqMan® chemistry, "S" denotes SYBR® Green chemistry

2.13 *In situ* immunodetection of recombinant AMPs in mature barley grains

Immunolabeling of recombinant antimicrobial peptides in mature grains of barley T0, T1 and T2 lines (in case of *HBD* and *PEX* expressing lines were analysed only T1 lines) was performed according to the published protocol (Qu *et al.*, 2003) with minor modifications. These included the blocking of the grain sections in 5% w/v bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 4 h.

The presence of recombinant LL-37 was visualised either with colorimetric or fluorescence probe. The grain sections were first incubated with 1:40 v/v LL-37 antiserum (sc-50423; Santa Cruz Biotechnology, USA) in TBS containing 2.5% w/v BSA for 90 min at 37 °C. The samples for colorimetric and fluorescence visualisation were then incubated at 20 °C for 90 min with 1:2500 v/v goat anti-rabbit alkaline phosphatase conjugate (AP307A, Merck) and 1:700 v/v Alexa Fluor 488 (A-11034; ThermoFisher Scientific), respectively. Fluorescence samples were protected from light until the analysis was completed.

Accumulation pattern of pexiganan was analysed with the rabbit polyclonal primary antibody (1:40 v/v; customized by the company EXBIO, Czech Republic) and the goat anti-rabbit secondary antibody (1:2500 v/v; A3687; Merck). For analysis of grains expressing *HBD2* transgene, the mouse monoclonal antiserum (1:40 v/v; ab66072, Abcam, UK) and the goat anti-mouse secondary antiserum (1:2500 v/v; A2429; Merck) were used.

For colorimetric detection of the recombinant AMPs, the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyphosphate substrate (B6404, Merck) was used. Images from colorimetric as well as fluorescence visualisaton were captured by Nikon SZM800 stereomicroscope.

2.14 Isolation of recombinant antimicrobial peptides from the plant material

For analysis of tobacco, the leaves were collected at the day 3 post infiltration and proteins were extracted with 0.02 M Tris-HCl pH 8.0 containing 0.15 M NaCl, 1 mM ethylenediaminetetraacetic acid (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 12 000 x*g* for 30 min, all at 4 °C.

For isolation of rAMPs from transgenic barley (of T0 and T1 generations), vegetative tissues and grains were harvested at the late milk developmental stage (BBCH 77). Furtherome, senescing barley grains (BBCH 99) were also analysed. Frozen plant tissues (of *rDEF, rPEX* and *rLL-37* expressing lines) were milled in MM 400 homogenizer (Retsch, Germany) with the grinding jars pre-cooled with liquid nitrogen. To choose the most appropriate method of protein extraction, several different experimental approaches listed below were tested. In all cases, protein content was estimated using the Bradford assay with a bovine serum albumin as standard, and the presence of rAMPs was analysed by Western blotting.

a. First isolation technique was based on the extraction of basic AMPs under acidic conditions. For this purpose, 0.15 g of plant material was mixed by vortexing with 300 µl of 0.1% trifluoroacetic acid containing cOmplete™ EDTA-free Protease Inhibitor Cocktail (Roche). This was followed by boiling the samples at 100 °C for 10 min and centrifugation at 12 000 x*g* for 30 min at 20 °C. The pellets were then re-extracted once again in the same way and the supernatant fractions were pooled and analysed.

b. The second experimental approach was based on the extraction with an ethylene glycol derivative using RIPA buffer and Buffer E, respectively. The chemical composition of the RIPA buffer (pH 7.5) was as follows: 20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, 150 mM NaCl, 1 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid), 1.0% NP-40, 1% sodium deoxycholate, and cOmplete™ EDTA-free Protease Inhibitor Cocktail. The Buffer E (pH 7.5) was composed of a mixture of 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 75 mM NaCl, 1 mM EGTA, 1mM $MgCl_2$, 1mM NaF, 10% glycerol, 1 mM DTT (dithiotreitol), and cOmplete™ EDTA-free Protease Inhibitor Cocktail. Samples were mixed by vortexing with either the RIPA buffer or the Buffer E at the ratio of 0.15 ml per 1 g of tissue and incubated for 15 min at either 4 °C or 70 °C. This was followed by centrifugation at 21 000 x*g* for 30 min at 4 °C for protein harvesting. Final separation step exploited differences in protein size using protein concentrator with 3 kDa cut-off (Merck Millipore, Germany).

c. The third extraction method was based on differences in solubility. In this case, milled plant material was extracted with four different solvents to obtain albumin, globulin, prolamin and glutelin fractions according to a previously described protocol (Chmelík *et al.*, 2002). Briefly, always 2 ml of four various extraction solvents were applied consecutively to 1 g of homogenized barley tissue. First, the samples were extracted for 20 min at 4 °C with distilled water and than centrifuged at 7 000 x*g* for 15 min at 4 °C to get the albumin fraction. Next, the samples were mixed with 5% (w/v) NaCl and the extraction was done again in the same way to get the globulin fraction. This was followed by extraction with 70% (v/v) ethanol and with 0.2% (w/v) NaOH to get the prolamin and glutelin fraction, respectively. Finally, the individual extracts were concentrated by freeze-drying prior to analyzing them by the use of Western blot.

d. Total soluble proteins were also 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. pH value of the buffers was set up to 8.0. The extraction was performed for 2 h at 4 °C, then the samples were centrifuged at 12 000 x*g* for 30 min at 4 °C. The pellets were re-extracted once more as described above, the supernatant fractions were combined and subsequently used for chromatographic separation (see the Chapter 2.15). Protein extracts isolated from plants expressing either the recombinant pexiganan, or chimeric versions of LL-37 lacking tags for afinity chromatography, were concentrated and partially purified using centrifugal filters with appropriate nominal molecular weight limit (3 kDa, 10 kDa, or 50 kDa; Merck Millipore, Germany), selected on the basis of isolated recombinant protein size.

e. Finally, to test whether the individual chimeric versions of rAMPs accumulate in protein storage organelles, protein-body enriched fraction was prepared. To do so, protein extracts from 200 mg of freshly collected vegetative tissues and grains in the late milk developmental phase as well as dry grains at the harvesting stage were prepared by slightly adapting the published method (Bundó *et al.*, 2014).

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 21 000 x*g* for 15 min at 4 °C. Protein samples of 0.2 mg were precipitated by acetone as described in chapter 2.15 of this thesis and subjected to immunoblotting analysis.

2.15 Chromatographic separation of recombinant antimicrobial peptides

Histidine-tagged versions of rLL-37 and the recombinant human beta-defensin 2 fused to 8xHis Tag were purified using the Co^{2+}- iminodiacetic acid (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. rLL-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 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 Western blot analysis, 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 xg 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.

2.16 Western blot analysis

All protein samples were separated under reducing conditions on 4 - 12% Bis-Tris Plus precast polyacrylamide gels (Thermo Fisher Scientific) and electroblotted to a polyvinylidene difluoride (PVDF) membrane (Merck Millipore).

Immunodetection of rLL-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.

PVDF membranes carrying the electroblotted proteins of *PEX* and *HBD2* expressing lines were first blocked with 5% (w/v) milk in TBS buffer (20 mM Tris/HCl, 500 mM NaCl, pH 7.5) containing 0.05% of Tween-20 (T), for 1 h. This was followed by incubation with either the rabbit polyclonal primary antibody raised against PEX (1:2000 v/v; EXBIO, CR), or the mouse monoclonal antibody against HBD2 (1:500 v/v; ab66072, Abcam, UK). The membranes were then rinsed once with TBS-T buffer (5 min) and twice with TBS buffer (2 x 5 min). Subsequently, the membranes were incubated with the secondary antibody in 1% (w/v) milk in TBS-T buffer for 1 h. For this purpose, either the goat anti-rabbit IgG containing conjugated alkaline phosphatase (1:5000 v/v; A3687; Merck, USA) or the mouse monoclonal antiserum (1:5000 v/v; ab66072, Abcam, UK) was used. The membranes were finally washed for 10 min once with TBS-T buffer and twice with TBS buffer.

Protein detection was performed using enhanced chemiluminescent (ECL) substrate (Bio-Rad). To determine the accumulation level of rLL-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 3 different biological replicates per line. The SeeBlue Plus2 Prestained Standard *(*Thermo Fisher Scientific) served as a molecular size marker.

2.17 Detection of rLL-37 by mass spectrometry

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 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).

2.18 Evaluation of antibacterial activity of rLL-37 peptide

Purified rLL-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 the final concentration of 10 µg/µl. 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. For cleavage efficiency analysis, the proteins were precipitated by acetone and analysed by Western blot analysis.

Antimicrobial activity of barley derived rLL-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 x 10^8 colony forming units (CFU)/ml) and 0.5 µl aliquots were mixed with 10 µl of purified fractions containing between 1 to 3 µg of rLL-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 rLL-37 and synthetic LL-37 to that of control. The bioassay was carried out in at least two independent experiments with three technical replicates.

3 Results

3.1 Generation of cloning cassettes harboring various *LL-37* genes for their expression in tobacco leaf tissues

Constructs for expressions of 10 different *rLL-37* variants in tobacco were prepared. All the commercially synthesized chimeric *LL-37* DNA sequences lacking both the promoter as well as the transcription terminator sequences were first cloned into the pENTR2B entry vector harboring *att*L1 and *att*L2 cloning sites. Plasmid DNA of selected *E.coli* TOP10 clones was subjected to control restriction digestion using *Apa*I endonuclease (Fig. 9). This was followed by creating novel genes including 35Sp::rLL-37::Nos-t in pGWB17 vector (Fig. 6) via Gateway LR recombination reaction between linearized pGWB17 and circular pENTR2B vectors possessing individual *rLL-37* variants. Presence of each complete cassette in selected surviving *E. coli* TOP10cells was confirmed by a digestion of isolated plasmid DNA with *Not*I enzyme (Fig. 10). Furthermore, after insertion of prepared cloning cassettes into *A. tumefaciens* strain GV3101, the identity of final costructs was checked by commertial sequencing service (Chapter 2.1).

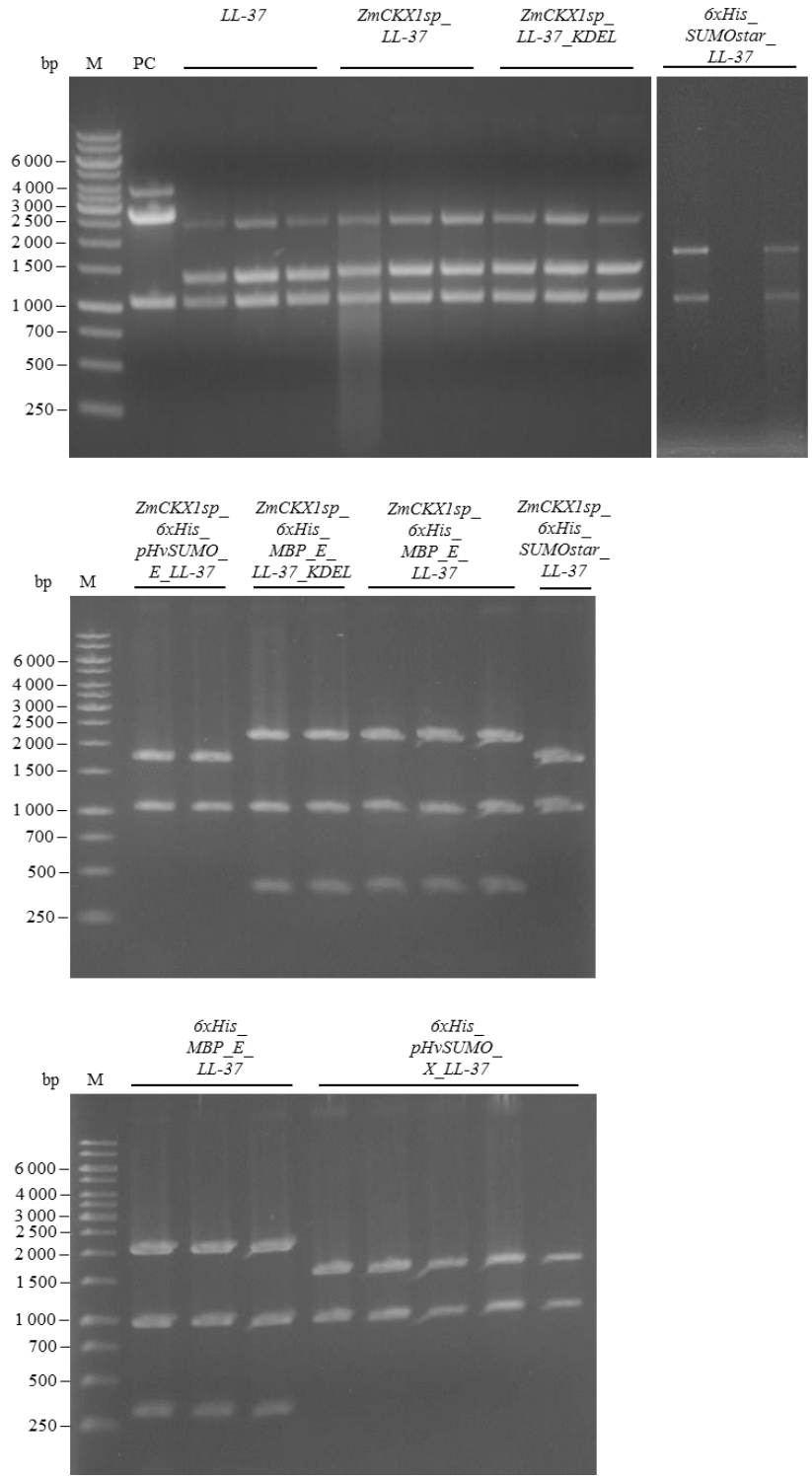

Figure 9. Restriction analysis of plasmid DNA (1 500-3 000 ng) of *E. coli* TOP10 cells harboring indicated transgenes in pENTR2B Gateway-compatible entry vector. Diagnostic digest was performed by *Apa*I enzyme. Expected fragment sizes (in bp) were as follows: *LL-37* - 1369 and 1054; *ZmCKX1sp_LL-37* - 1420 and 1054; *ZmCKX1sp_LL-37_KDEL* - 1432 and 1054; *6xHis_SUMOstar_LL-37* - 1693 and 1054; *ZmCKX1sp_6xHis_pHvSUMO_E_LL-37* - 1741 and 1054; *ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL* - 2207, 1054, and 407; *ZmCKX1sp_6xHis_MBP_E_LL-37* - 2195, 1054, and 407;

ZmCKX1sp_6xHis_SUMOstar_LL-37 - 1744 and 1054; *6xHis_MBP_E_LL-37* - 2195, 1054, and 356; *6xHis_pHvSUMO_X_LL-37* - 1699 and 1054; pENTR2B dual selection vector (PC) - 2701 bp and 1054. M, DNA size marker.

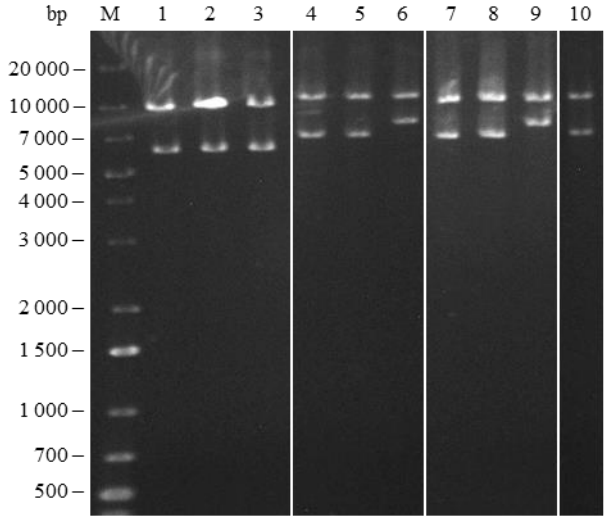

Figure 10. Restriction analysis of plasmid DNA (500-1 000 ng) of PCR positive *E. coli* TOP10 cells harboring various chimeric *LL-37* genes in pGWB17 Gateway-compatible destination vector. Diagnostic digest was performed by *Not*I enzyme. Expected fragment sizes (in bp) were as follows: *LL-37* - 9813 and 6121 (1); *ZmCKX1sp_LL-37* - 9813 and 6172 (2); *ZmCKX1sp_LL-37_KDEL* - 9813 and 6184 (3); *ZmCKX1sp_6xHis_SUMOstar_LL-37* - 9813 and 6496 (4); *6xHis_SUMOstar_LL-37* - 9813 and 6445 (5); *ZmCKX1sp_6xHis_MBP_E_LL-37* - 9813 and 7354 (6); *6xHis_pHvSUMO_X_LL-37* - 9813 and 6409 (7); *ZmCKX1sp_6xHis_pHvSUMO_E_LL-37* - 9813 and 6451 (8); *ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL* - 9813 and 7366 (9); *6xHis_MBP_E_LL-37* - 9813 and 7303 (10). M, DNA size marker 1 kb Plus (5 µl, Thermo Scientific, USA).

3.2 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 agroinfiltrated with 5 randomly selected *rLL-37* DNA constructs and levels of transgene mRNA were relatively quantified by qPCR analysis using primers listed in Table 8. Amplification efficiency that was calculated from standard curves for individual genes showed 103.3% for *LL-37* expressing lines (primer assigned as 8), 103.9% for analysis of *ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL* expressing lines (primer assigned as MBP), 111.4% for analysis of *ZmCKX1sp_6xHis_SUMOstar_LL-37*

and *6xHis_SUMOstar_LL-37* expressing lines (primer assigned as SUMOstar), and 95.64% for analysis of tobacco elongation factor 1 (primer assigned as NbeEF1). High accumulation levels of transgene transcripts were detected 2-4 days post-inoculation but significantly decreased afterwards (Fig. 11). Accordingly, the expression of all of the 10 designed chimeric *LL-37* genes was analysed on the third day after infiltration (Fig. 12A). Although RT-PCR analysis confirmed the accumulation of transgenic mRNA in all samples (Fig. 12B), Western blot analysis showed the presence of rLL-37 peptide only for 3 out of the 10 constructs as depicted in Fig. 12C. 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 rLL-37 peptide accumulation in plant tissue. Based on this observation, the three positive *rLL-37* peptide gene variants, *ZmCKX1sp_LL-37*, *ZmCKX1sp_LL-37_KDEL* and *ZmCKX1sp_6xHis_MBP_PRO_LL-37_KDEL* (Fig. 6B; amino acid sequences are shown in Supporting information, Fig. S1BCF) were selected for the heterologous expression in barley. Moreover, two additional variants *ZmCKX1sp_$(GGGGS)_2$_6xHis_$(GGGGS)_2$_E_LL-37* and *OsCht1sp_6xHis_E_LL-37* consisting of gene coding for ER transit peptide *ZmCKX1sp* or *OsCht1sp* (rice chitinase 1, GenBank: D16221.1), enterokinase recognition sequence (*E*) and *6xHis* tag, which was in one case flanked by gene for a flexible linker sequence *$(GGGGS)_2$* to ensure effective separation of the domains (see the amino acid sequences in Supporting information, Fig. S1KL), were designed.

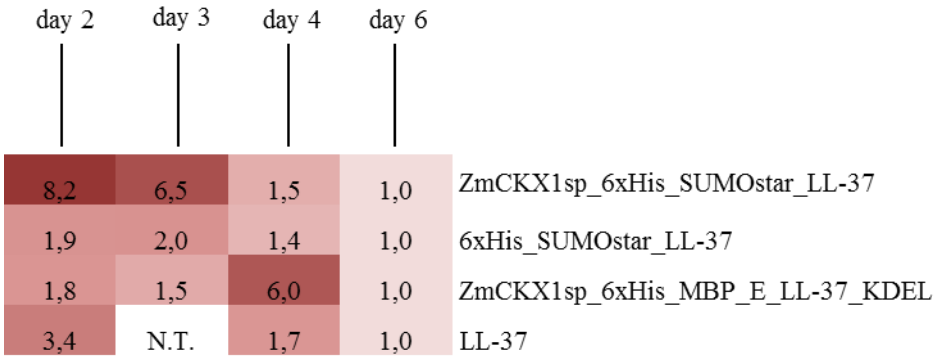

Figure 11. Temporal assesment of expression levels of indicated transgenes in tobacco leaf tissues infiltrated by pGWB17 destination vectors harboring relevant sequences. Expression levels of target genes were relatively quantified using the DataAssist™ software. The experiment was carried out for 3 different biological replicates and in 3 technical repeats for each transformation event. N.T. not tested.

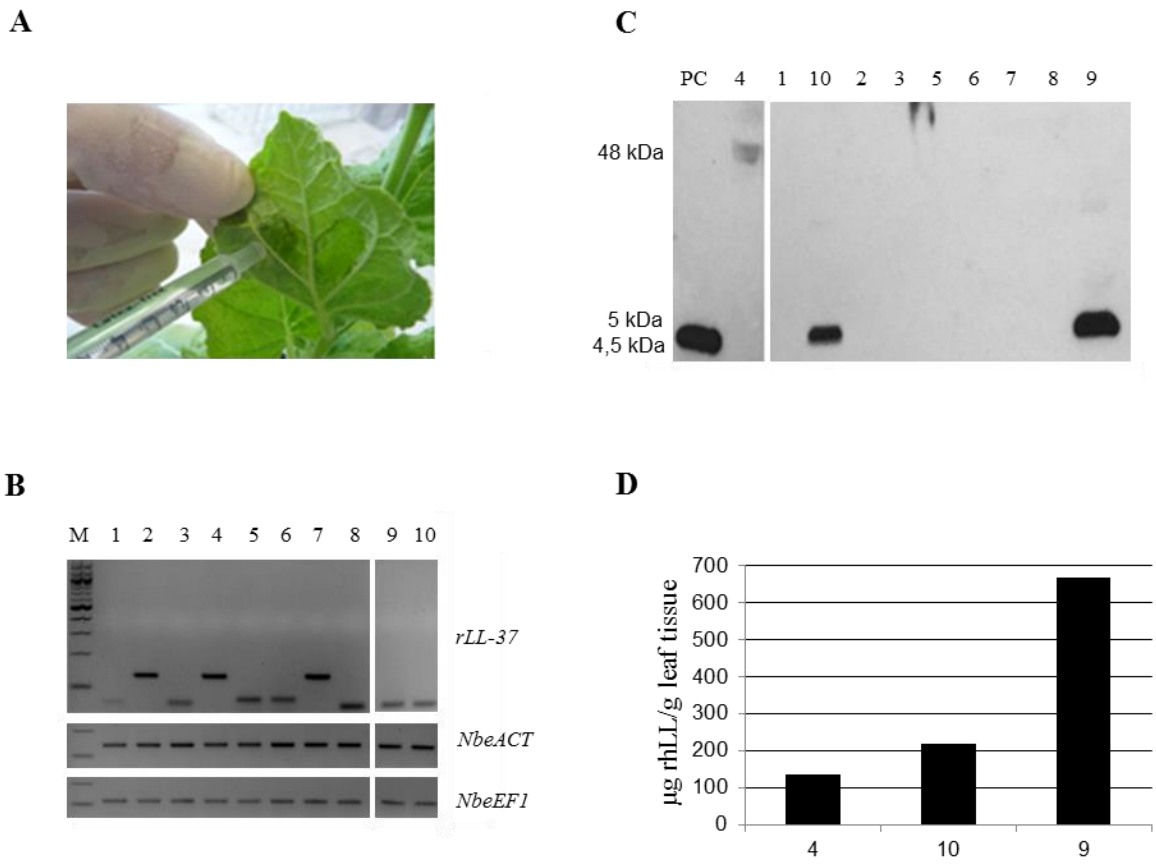

Figure 12. Transient expression analysis of chimeric *LL-37* genes in tobacco *Nicotiana benthamiana L.* (*Nbe*) infiltrated with *Agrobacterium* bearing 10 different expression vectors: 1, *6x His_SUMOstar_LL-37*; 2, *ZmCKX1sp_6x His_MBP_PRO_LL-37*; 3, *ZmCKX1sp_6x His_SUMOstar_LL-37*; 4, *ZmCKX1sp_6x His_MBP_PRO_LL-37_KDEL*; 5, *ZmCKX1sp_6x His_pHvSUMO_PRO_LL-37*; 6, *6x His_pHvSUMO_PRO_LL-37*; 7, *6x His_MBP_PRO_LL-37*; 8, *LL-37*; 9, *ZmCKX1sp_LL-37*; 10, *ZmCKX1sp_LL-37_KDEL*. Infiltrated area of four leaves was pooled and analysed. (A) Syringe infiltration of *Nicotiana benthamiana* leaf. (B) RT-PCR amplification of chimeric genes coding for recombinant human LL-37 (*rLL-37*), and tobacco actin (*NbeACT*) and elongation factr 1 (*NbeEF1*) as loading controls. Sequences of individual primers and expected amplicon sizes are summarized in Table 8. M, 100 bp DNA size marker. (C) Immunoblot analysis of total soluble proteins. PC, synthetic LL-37 antimicrobial peptide (10 ng). (D) Accumulation of the recombinant LL-37 products in tobacco fresh leaf tissues as estimated by Western blot analysis of crude protein extracts in comparison with known amounts of synthetic LL-37 peptide.

3.3 Selection of barley endosperm specific promoters for molecular farming purposes

Based on results obtained through Genevestigator platform, 5 barley endogenous genes with endosperm-preferred expression were selected as the most promising candidates to drive tissue-specific accumulation of rLL-37 in grains (Fig. 13A). The considered candidate genes included: *B Hor* (B1 hordein, GenBank: X87232.1), *D Hor* (D hordein, GenBank: X84368.1), *Hinb* (hordoindoline b, GenBank: AY644004.1), *CI2* (chymotrypsin inhibitor 2, GenBank: X57035.1), and *TI* (trypsin inhibitor, GenBank: X65875.1). Expressions of the genes were compared using qPCR analysis with primers listed in Table 4. The efficiency of amplification was calculated from standard curves for individual genes as 98.0% for B1 hordein (primer assigned as B-Hor), 93.8% for D hordein (D-Hor), 103.1% for hordoindoline b (Hinb), 97.8% for chymotrypsin inhibitor 2 (CI2), and 96.4% for trypsin inhibitor (TI). As shown in Figure 13B, the barley B1 hordein gene promoter revealed the strongest expression levels of corresponding endogenous gene in later developmental stages of wild-type barley spikes. Furthermore, the *B Hor* gene promoter was used to direct stable overexpression of cytokinin oxidase/cytokinin dehydrogenase 9 gene (*HvCKX9*), where the tissue specific gene delivery was found in T2 homozygous barley plants using qPCR assay. The relative transcript levels of this cytokinin metabolizing gene were determined in barley endosperm, embryo, aleurone, and seed coat tissues. Raised expression of *HvCKX9* gene was observed in all transgenic samples tested. However, the predominant overexpression of transgene was observed in endosperm tissue, with the relative transcript level being approximately 32 500 times increased compared to non-transgenic control plants. Increase in transgene expression in the rest of the tissues was fairly similar, with the relative transcript abundance being approximately 4 800 times higher in embryo, 2 600 higher in aleurone, and 1 000 higher in seed coat tissue. Hence, the barley B1 hordein gene promoter exhibits high level of endosperm specificity (Figure 14). Additionaly, the aforementioned T2 homozygous transgenic barley lines were also used for analysis of B1 hordein gene promoter activity dynamics via determining of *in vitro* CKX/CKO activity in grains of various growth stages. As shown in Figure 15, all of the 3 independent transgenic lines analysed showed similar characteristics of temporal changes in specific CKX/CKO activity during the growth and development transgenic barley, with highest increase (up to 1985% for line 1, 1544% for line 2 and 2735% for line 3, respectively)

in the early dough grains (GS83). Opposite to that, CKX enzyme activity in the flag leaf sheath extending growth stage (GS41), first awns visible growth stage (GS49) and the complete emergence of ear above flag leaf ligule growth stage (GS59) was proportional to that measured in control non-transgenic *in vitro* regenerated plants. The obtained data showed, that B1 hordein gene promoter is predominantly active in the endosperm at the mid and late stages of barley grain development, which is beneficial for molecular farming purposes in cereal grains. Thereby, the *B Hor* promoter was selected to drive controlled endosperm-specific accumulation of most of the AMP variants expressed in barley in scope of this thesis (see Figure 7C). In addition, promoter of trypsin inhibitor gene was also selected for driving the endosperm specific accumulation of *rPEX* (Figure 8A). The expression profile of its corresponding endogenous gene showed strong and strictly endospem-specific accumulation (see Figure 13A), but it seems to be predominantly active in later stages of barley seed growth and develoment (Fig. 13B).

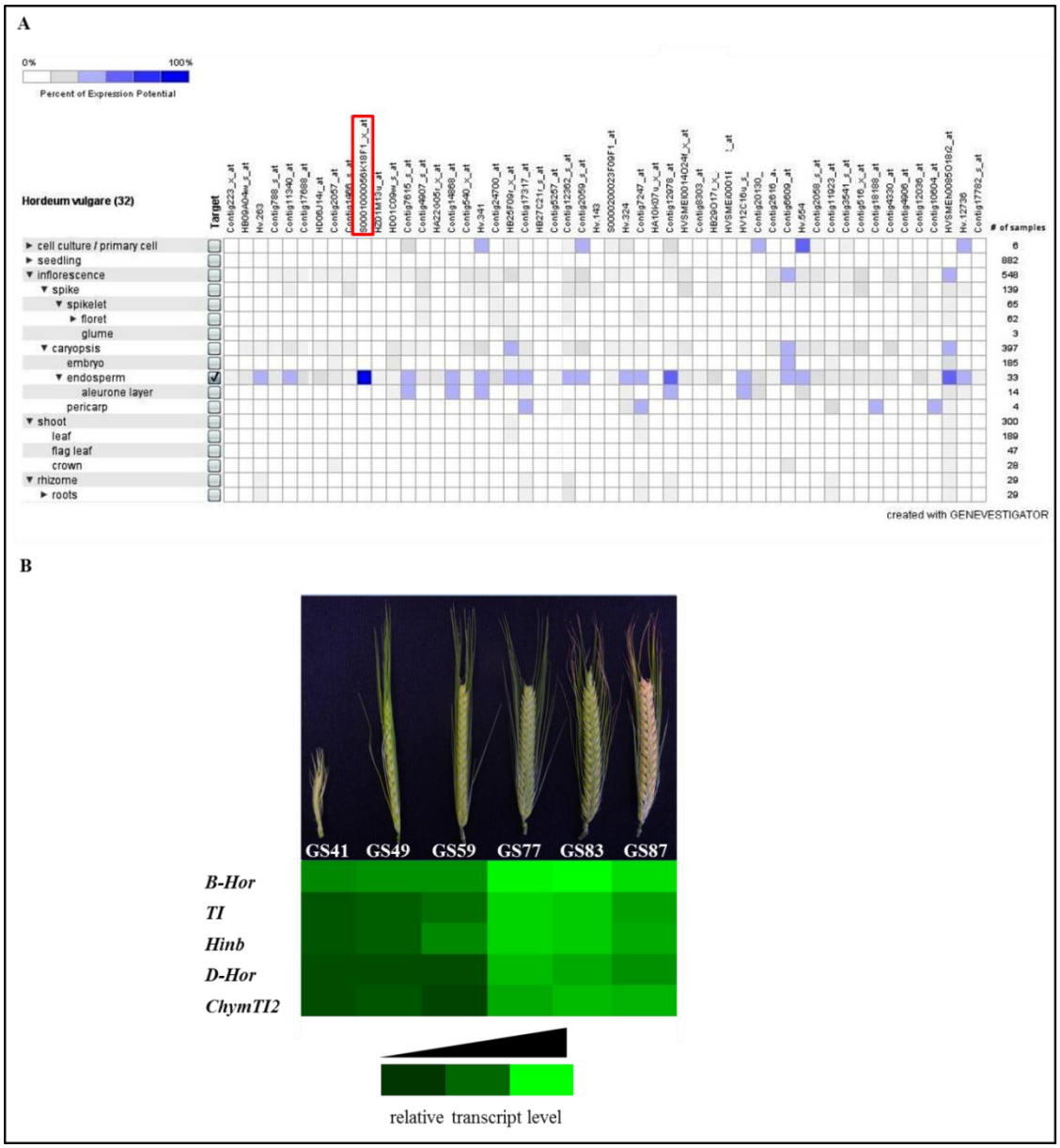

Figure 13: Results from GENEVESTIGATOR search (A) and temporal expression profiles of selected barley genes (B). B1 hordein (*B Hor*), trypsin inhibitor (*TI;* contig enclosed in red rectangle), hordoindoline b (*Hinb*), D hordein (*D Hor*) and chymotrypsin inhibitor 2 (*CI2*) genes were shown to be preferably expressed in endosperm (A). Temporal control of expression driven by their native promoters was analysed as the relative transcript levels by qPCR using ΔΔCt relative quantification method corrected by an efficiency factor (B).

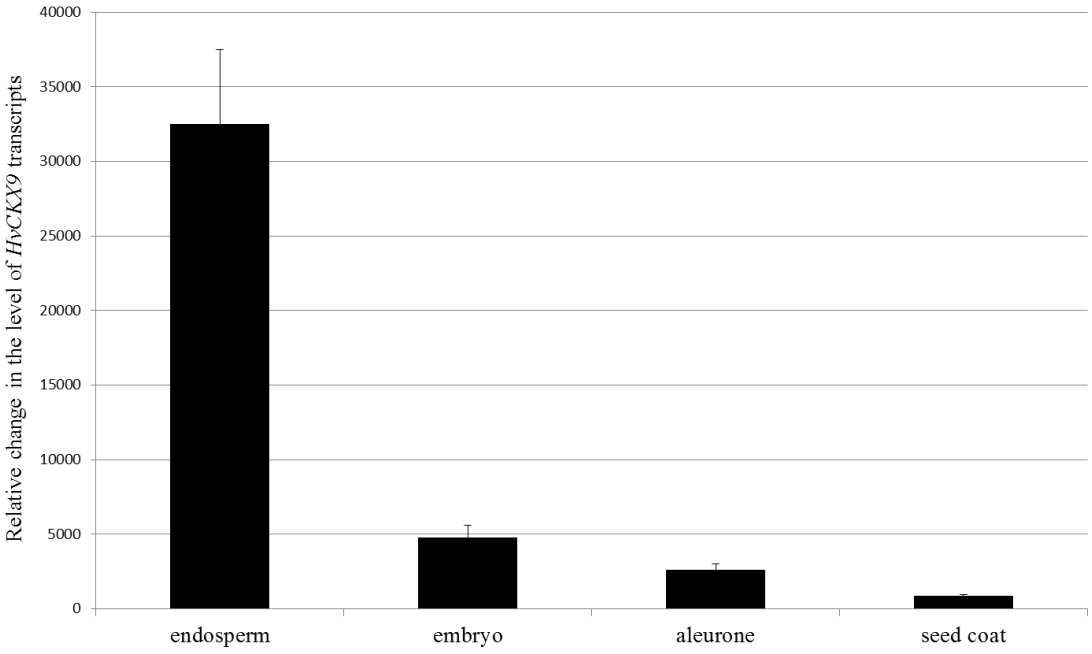

Figure 14. qPCR analysis of tissue specific gene delivery of B1 hordein gene promoter by determining relative changes in *HvCKX9* transcript level in late milk endosperm grains (GS77) of T2 homozygous transgenic barley lines with integrated BRACT209/*HORp::HvCKX9::Nos-t* expression cassette. Error bars represent the standard deviation of 3 independent transgenic lines each including at least 3 plants. The DataAssist^{TM} software was used to evaluate the data using the ΔΔCt method corrected by an efficiency factor. Expression is presented relative to that in the control non-transgenic tissue culture regenerated plants, which was arbitrarily set to the value of 1. For primers used see Table 4.

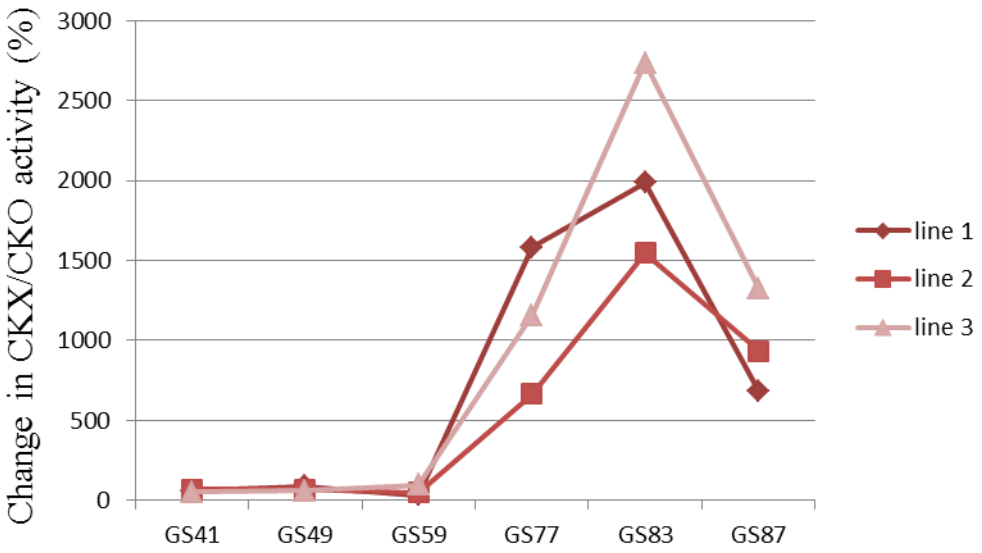

Figure 15. Analysis of temporal activity of B1 hordein gene promoter by determining of specific CKX/CKO (cytokinin oxidase/dehydrogenase) activity in crude protein extracts prepared from spikes of T2 homozygous transgenic barley lines with integrated pBRACT209/*HORp::HvCKX9::Nos-t* expression cassette. CKX/CKO activity was determined for 3 independent lines, always in 4 biological and 2 technical replicates. Specific CKX/CKO activities in each developmental stage are shown as relative values to those of control *in vitro* regenerated plants, which were set to 1 (100%).

3.4 Generation of cloning cassettes harboring various *LL-37* genes for their expression in barley

For stable expression of recombinant LL-37 peptides in barley, 8 binary vectors were prepared and subsequently used for the transformation. As depicted in Figure 7, 5 out of the 8 cloning cassettes were designed for a grain specific expression of *rLL-37* driven by the B1 hordein gene promoter (Fig. 7C), and other 3 cassettes were prepared for a constitutive expression of various *rLL-37* genes driven by the maize ubiquitin promoter (Fig. 7D).

The constructs for a grain specific expression of *rLL-37* were successfully cloned into the pENTR 1A Dual Selection Vector and identity of each of the gene cassettes, consisting of B-HORp::rLL-37::Nos-t, was confirmed by control restriction analysis using *Xho*I and *Dra*I enzymes (Figure 16). Afterwards, the genes were subcloned into the pBRACT209 vector as already described in chapter 2.4 of this thesis. The correct insertion of the genes into the destination vector was confirmed by PCR analysis of *E. coli* TOP 10 cells (Fig. 17) as well as by restriction analysis of plasmid DNA from the PCR positive clones (Fig. 18).

In addition, a series of constructs for ubiquitous expression of chimeric *LL-37* was successfully created. In detail, *rLL-37* genes were subcloned from the pENTR2B vector (see Fig. 4; note: cloning of *ZmCKX1sp_(GGGGS)$_2$_6xHis_(GGGGS)$_2$_E_LL-37* into pENTR2B vector was done in the academic year 2013/2014 by Alžbeta Mičúchová during her Summer biotechnology project 1 under my supervision) into the pBRACT214 destination vector and correct insertion and orientation of inserted genes was confirmed by PCR analysis (Figure 19), control restriction reaction (Figure 20) and sequencing by a commercial service as already stated in the Chapter 2.4.

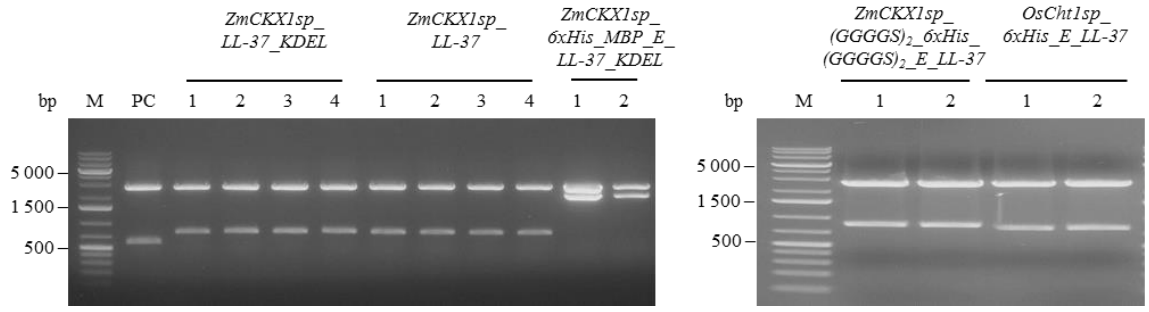

Figure 16. Control restriction analysis of plasmid DNA of PCR positive *E. coli* TOP10 cells harboring B-HORp::rLL-37::Nos-t in pENTR1A Dual Selection Vector. Restriction enzyme digestion was performed by *Xho*I and *Dra*I enzymes. Expected fragment sizes (in bp) were as follows: *ZmCKX1sp_LL-37_KDEL* - 2 567 and 764; *ZmCKX1sp_LL-37* - 2 567 and 752; *ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL* - 2 567 and 1 946; *ZmCKX1sp_(GGGGS)$_2$_6xHis_(GGGGS)$_2$_E_LL-37* - 2 567 and 840; *OsCht11sp_6xHis_E_LL-37* - 2 567 and 800; *B-HORp::empty::Nos-t* in pENTR1A Dual Selection Vector - 2 567 and 584 (PC, 250 ng). M, DNA size marker 1 kb Plus (5 µl, Thermo Scientific, USA); 1 - 4, transgenic *E. coli* cells harboring indicated seqeuences.

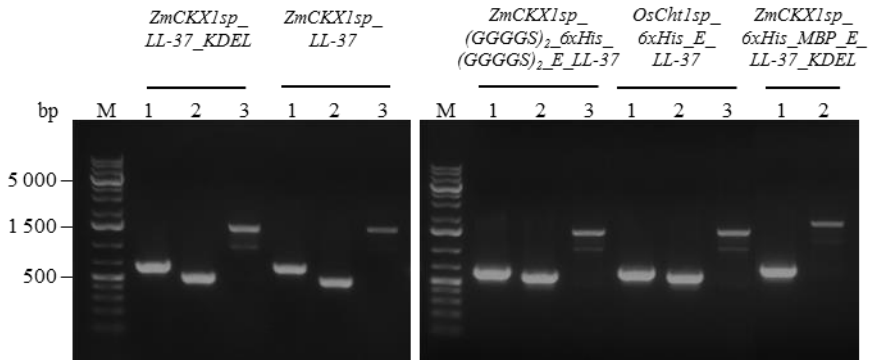

Figure 17. PCR analysis of plasmid DNA confirming the presence of selected functional fragments in *E. coli* TOP 10 cells after recombination of *B-HORp::rLL-37::Nos-t* in pENTR1A to pBRACT209 Gateway-compatible destination vector. Individual *rLL-37* gene variants are inditaced. (1) Amplification of the *hpt* gene produced a fragment of expected size 649 bp for all constructs used. (2) Amplification of part of the *LL-37* gene plus part of the *B-HORp* of expected fragment sizes (in bp): *ZmCKX1sp_LL-37_KDEL* - 499; *ZmCKX1sp_LL-37* - 499; *ZmCKX1sp_(GGGGS)$_2$_6xHis_(GGGGS)$_2$_E_LL-37* - 592; *OsCht11sp_6xHis_E_LL-37* - 538; *ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL* - 1 681. (3) Amplification of part of the *hpt* gene fragment plus part of the *LL-37* gene of expected fragment sizes (in bp): *ZmCKX1sp_LL-37_KDEL* - 1 438; *ZmCKX1sp_LL-37* - 1 426; *ZmCKX1sp_(GGGGS)$_2$_6xHis_(GGGGS)$_2$_E_LL-37* - 1 519. M, DNA size marker 1 kb Plus (5 µl, Thermo Scientific, USA).

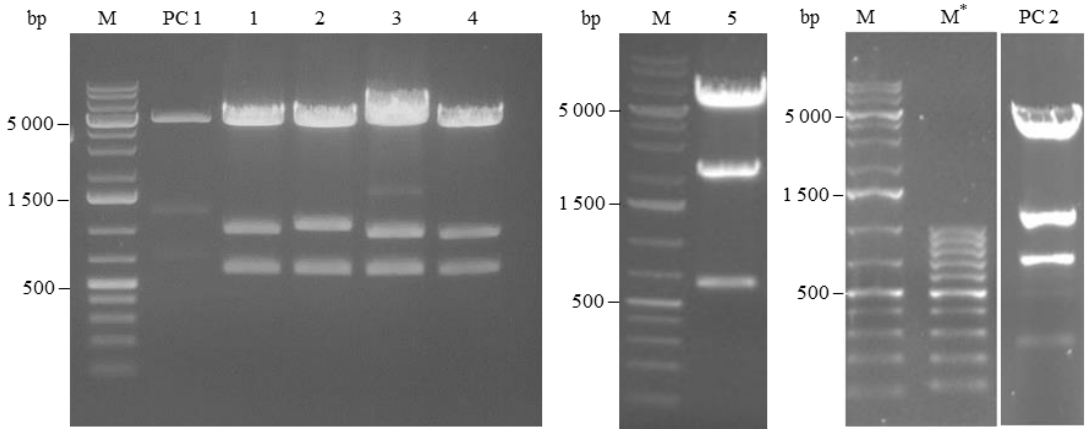

Figure 18. Restriction analysis of plasmid DNA of PCR positive *E. coli* TOP10 cells harboring B-HORp::rLL-37::Nos-t in pBRACT209 Gateway-compatible destination vector. Diagnostic digest was performed by *BamH*I enzyme. Expected fragment sizes (in bp) were as follows: *B-HORp::OsCht11sp_6xHis_E_LL-37::Nos-t* - 4 757, 978 and 612 (1); *B-HORp::ZmCKX1sp_(GGGGS)$_2$_6xHis_(GGGGS)$_2$_E_LL-37::Nos-t* - 4 757, 1032 and 612 (2); *B-HORp::ZmCKX1sp_LL-37_KDEL::Nos-t* - 4 757, 951 and 612 (3); *B-HORp::ZmCKX1sp_LL-37::Nos-t* - 4 757, 939 and 612 (4); *B-HORp::ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL::Nos-t* - 4 757, 2 133 and 612 (5); empty pBRACT209 vector - 4 757, 1203, 703 and 209 (PC 1 and PC 2, respectively). PC 1 and PC 2 differed in the amount of plasmid DNA used for restriciton digest, which was 50 and 500 ng, respectively. M, DNAize marker 1 kb Plus (5 µl, Thermo Scientific, USA); M*, DNA size marker 100 bp (5 µl, Thermo Scientific, USA).

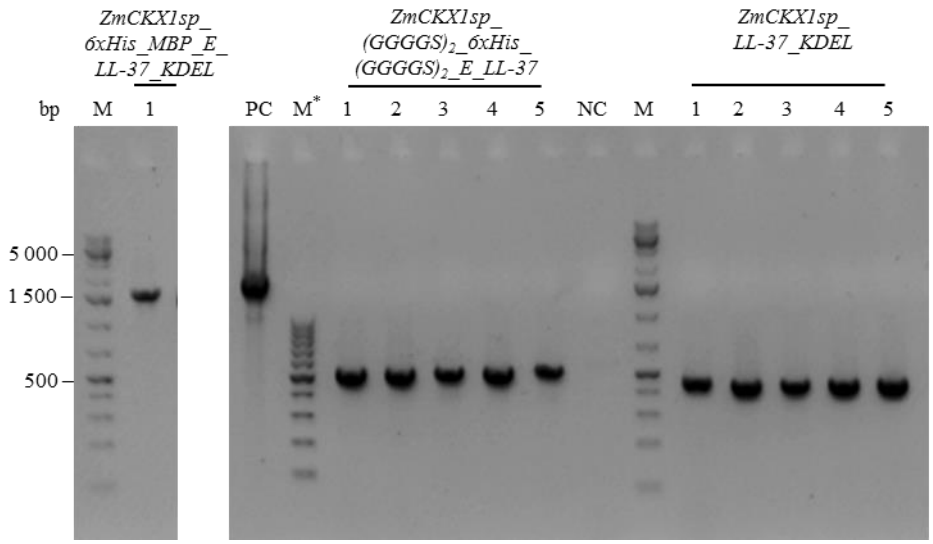

Figure 19. Colony PCR analysis of *E. coli* TOP10 clones confirming the presence of the indicated *LL-37* variants after recombination into pBRACT214 Gateway-compatible destination vector. Primers used for the analysis were compatible to the part of the *UBIp* and the *Nos-t*. Expected amplicon size of the empty pBRACT214 vector was 1844 bp (amplicon included part of the *UBIp*, attR1, chloramphenicol resistance gene, ccdb gene, attR2 and part of the *Nos-t*). Sizes of amplicons (in bp) after recombination of individual *LL-37* genes were as follows: *ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL* - 1628; *ZmCKX1sp_(GGGGS)$_2$_6xHis_(GGGGS)$_2$_E_LL-37* - 527; *ZmCKX1sp_LL-37_KDEL* - 446 (amplicons included part of the *UBIp*, attB1, *rLL-37* and attB2). M, DNA size marker 1 kb Plus (5 µl, Thermo Scientific, USA); M*, DNA size marker 100 bp (5 µl, Thermo Scientific, USA); PC, positive control of the PCR reaction (empty pBRACT214 plasmid); NC, negative control of the PCR reaction (nuclease-free water); 1 - 5, transgenic *E. coli* cells harboring *UBIp::rLL-37::Nos-t* in pBRACT214 expression vectors.

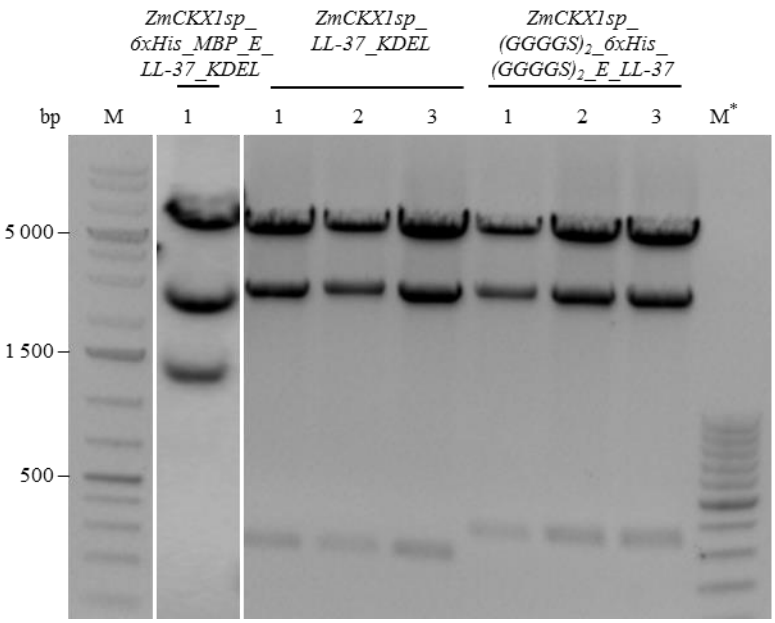

Figure 20. Restriction analysis of plasmid DNA of PCR positive *E. coli* TOP10 cells harboring *UBIp::rLL-37::Nos-t* in pBRACT214 Gateway-compatible destination vector. Diagnostic digest was performed by *BamH*I enzyme. Expected fragment sizes (in bp) were as follows: *UBIp::ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL::Nos-t* - 5 069, 2 440 and 1 440; *B-HORp::ZmCKX1sp_LL-37_KDEL::Nos-t* - 5 069, 2 440 and 258; *B-HORp::ZmCKX1sp_(GGGGS)$_2$_6xHis_(GGGGS)$_2$_E_LL-37::Nos-t* - 5 069, 2 440 and 339. M, DNA size marker 1 kb Plus (5 µl, Thermo Scientific, USA); M*, DNA size marker 100 bp (5 µl, Thermo Scientific, USA).

3.5 Generation of cloning cassettes harboring *rPEX* or *rHBD2* genes for their expression in barley

Based on an extensive search in the literature, the oat globulin promoter was selected as a promissing candidate that is able to drive strong endsperm-specific expression in cereal seeds and therefore represents an ideal promoter for biotechnology applications (Vickers *et al.*, 2006). Accordingly, this promoter sequence was exploited to drive tissue specific accumulation of the *rHBD2* gene (Fig. 8B). A grain specific accumulation of the *rPEX* was driven by the trypsin inhibitor gene promoter, whose stregth and dynamics were also examined (Fig. 8A). Corresponding sequences including promoter::rAMP::Nos-t (for details see the Chapter 2.5) were successfully inserted into pBRACT209 cloning vector, which was verified by colony PCR (Fig. 21) with the use of primers specific to hygromycin (*hpt*) gene fragment (part of the pBRACT209 destination vector). To further confirm correct insertion of the cloned genes, plasmid DNA of PCR positive clones was checked by restriction analysis with

the use of *Sal*I endonuclease (Fig. 22). Identities of final vectors were confirmed by DNA sequencing as in the case of analysis of *rLL-37* cloning vectors.

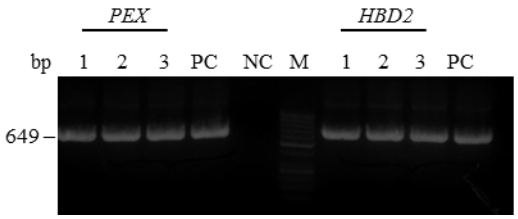

Figure 21. Amplification of the *hpt* gene fragment in *E. coli* TOP10 cells harboring *TIp::PEX::Nos-t/AsGLO1p::HBD2::Nos-t* in pBRACT209 destination vector by colony PCR. Expected fragment size was 649 bp for both of the constructs used. M, DNA size marker 50bp (2.5 µl, New England Biolabs (USA); PC, positive control of the PCR reaction (empty pBRACT plasmid, 100 ng); NC, negative control of the PCR reaction (nuclease-free water); 1 - 3, transgenic *E. coli* cells harboring *TIp::PEX_KDEL::Nos-t* (*PEX*) or *AsGLO1p::8xHis_E_HBD2::Nos-t* (*HBD2*) in pBRACT209 expression vector.

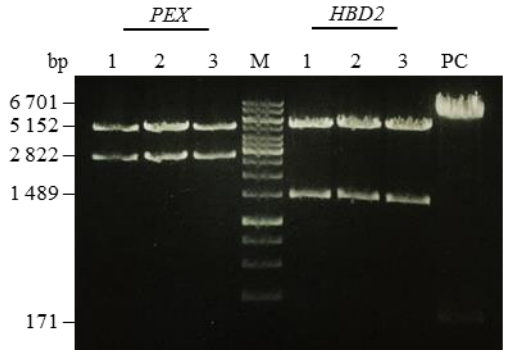

Figure 22. Restriction analysis of plasmid DNA of PCR positive *E. coli* TOP10 clones harboring *TIp::PEX::Nos-t/AsGLO1p::HBD2::Nos-t* in pBRACT209 destination vector. Restriction enzyme digestion was performed by *Sal*I enzyme. Expected fragment sizes were as follows: 5 152 bp + 2 822 bp for chimeric pexiganan gene in pBRACT209 (*PEX*), 5 152 bp + 1489 bp for chimeric human beta-defensin 2 gene in pBRACT209 (*HBD2*), and 6 701 bp + 171 bp for empty pBRACT209 (PC, 500 ng). M, DNA size marker 1 kb (5 µl, New England Biolabs, USA); PC, positive control of the restriction reaction (empty pBRACT209 plasmid); 1 - 3, transgenic *E. coli* cells harboring *TIp::PEX_KDEL::Nos-t* (*PEX*) or *AsGLO1p::8xHis_E_HBD2:: Nos-t* (*HBD2*) in pBRACT209 expression vector, respectively.

3.6 Production of stable barley transgenic lines for the expression of rAMPs

In total, 162 independent T0 stable transgenic barley lines expressing genes coding for various rAMPs were prepared using agroinfection of wounded immature barley embryos basically following the protocol of Harwood *et al.* (2009) as already described in Chapter 2.6 (Figure 23). There were 143 out of the 162 T0 plants prepared with the aim to express various *rLL-37* gene variants either under the *B-HORp* (Fig. 7C; note: transformation of barley by vectors harboring *ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL*, *OsCht11sp_6xHis_E_LL-37*, and *ZmCKX1sp_(GGGGS)$_2$_6xHis_(GGGGS)$_2$_E_LL-37* genes was performed by Mgr. Alžbeta Mičúchová during her bachelor and master thesis work supervised by myself) or the *UBIp* (Fig. 7D; cloning of *ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL*, and *ZmCKX1sp_LL-37_KDEL* genes was done by Mgr. Alžbeta Micúchová as stated above). Besides this, there were another 12 T0 lines with integrated chimeric *PEX* gene, and finally 7 T0 lines harboring r*HBD2* transgene prepared and analysed. The total number of inoculated embryos reached approximately 3 300. Hence, the overall transformation efficiency expressed as the number of independently transformed T0 plants per 3 300 inoculated embryos was about 5%. For further propagation to subsequent generations, only diploid T0 plants harboring either single or low copy number of T-DNA inserts were selected (see below).

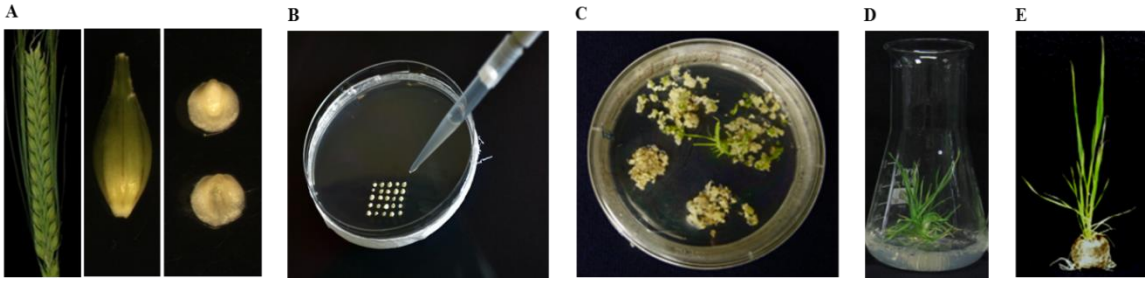

Figure 23. Generation of stably transformed barley lines in brief. (A) Selection of suitable barley spike containing immature grains at the proper developmental stage with the detail showing immature embryo with and without embryogenic axis. (B) Inoculation of injured immature barley embryos with *Agrobacterium tumefaciens* suspension culture carrying the effector transgenic T-DNA. (C) Selection of transgenic calli on medium containing hygromycin and shoot regeneration. (D) Regenerated transgenic shoots on medium with selection agens. (E) Regenerated transgenic plant growing in hydrated peat jiffy pellet.

3.7 Phenotype of transgenic barley lines

Except of the 1 line (with integrated *B-HORp::ZmCKX1sp_LL-37::Nos-t* gene), all T0 transgenic plants were fertile.

Heterologous expression of *LL-37* variants was not phytotoxic, as no apparent negative impact on plant development and phenotype resulting from the transgenic expression of *rLL-37* genes was observed in most cases under controlled environmental conditions in growth chambers or in greenhouse (Fig. 24).

Analysis of morphology of T1 *rHBD2* expressing lines showed somehow dissimilar results depending on the number of integrated transgenes. Single copy insertion T1 lines (in total 3 independent lines analysed) showed elongated stem with respect to the control plants, their growth rate was comparable to that of control plants and their seeds showed normal phenotype. The aforementioned phenomena were observed also in T2 homozygous single transgene insertion lines. Contrary to that, insertion of T-DNA in multiple copies (only 1 T1 line analysed) resulted in delayed development under greenhouse conditions (approximetly by one month), T1 plants were similar in hight as control plants, and their seeds showed enlarged endosperm cavities (Fig. 25).

All of the T1 *rPEX* expressing lines showed analogous morphology of grains as the control lines and the rate of their growth was comparable. Although single copy T-DNA insertion lines (3 lines analysed in total) were similar in height as *in vitro* regenerated non-transgenic plants, T1 plants with transgene integrated in more than 3 copies (only 1 line analysed) showed decreased stem length by approx. 20% (Fig. 26).

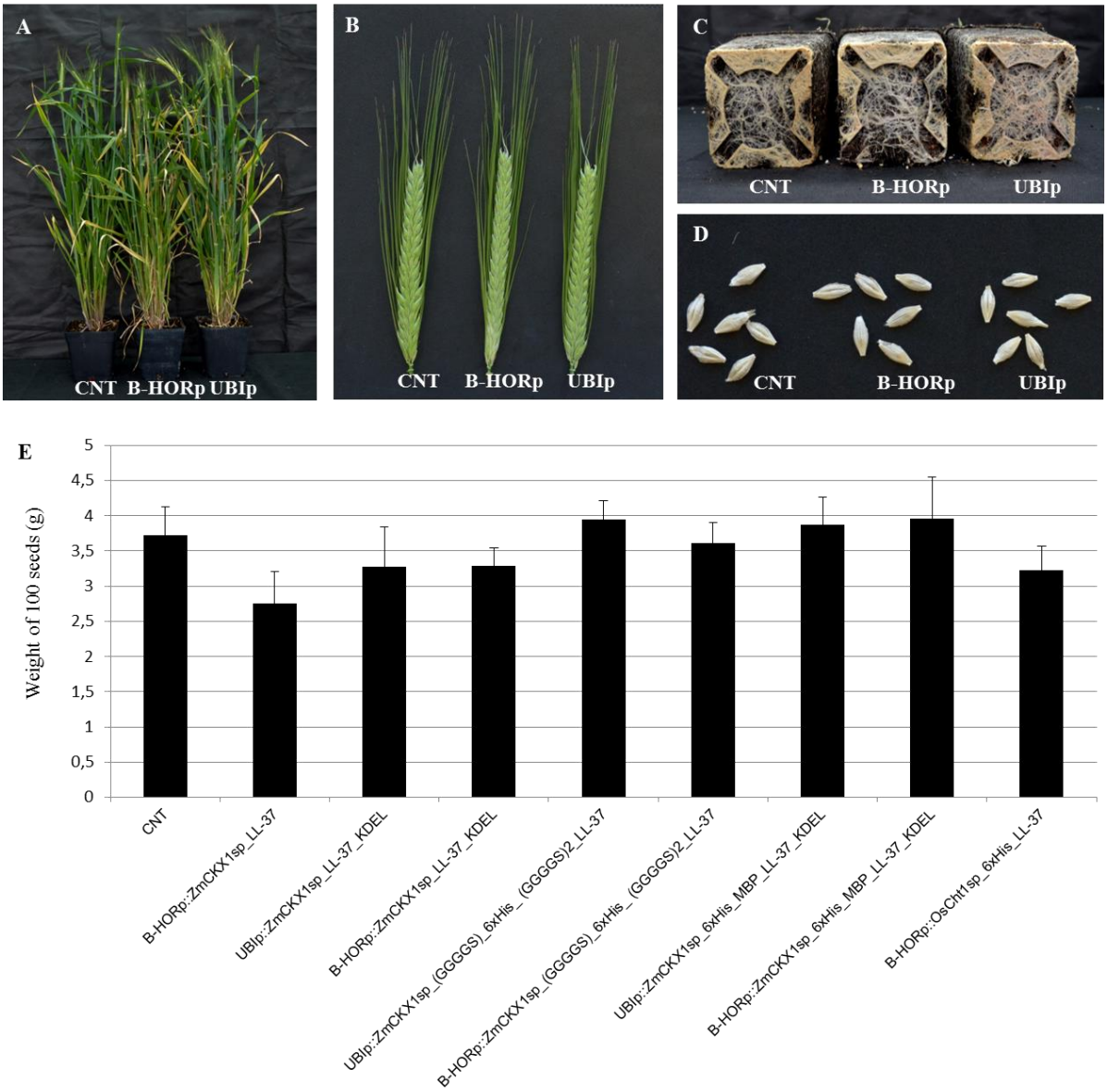

Figure 24. Comparison of phenotype of T1 transgenic barley plants expressing rLL-37 under grain specific B1 hordein gene promoter (*B-HORp*) or the maize ubiquitin gene promoter (*UBIp*) to untransformed tissue culture regenerated barley plants (CNT). Representative photos of the aerial part (A), spikes (B), roots (C), and mature grains (D) are displayed. (E) Yield parameters of transgenic lines, average weights of 100 mature grains of transgenic barley plants carrying the individual transgenes are indicated. Error bars represent the standard deviation of 3 independent lines per transformation event and at least 3 plants per line (with the exception of line *B-HORp::ZmCKX1sp_LL-37_KDEL*, in which 10 plants per only 1 line were analysed).

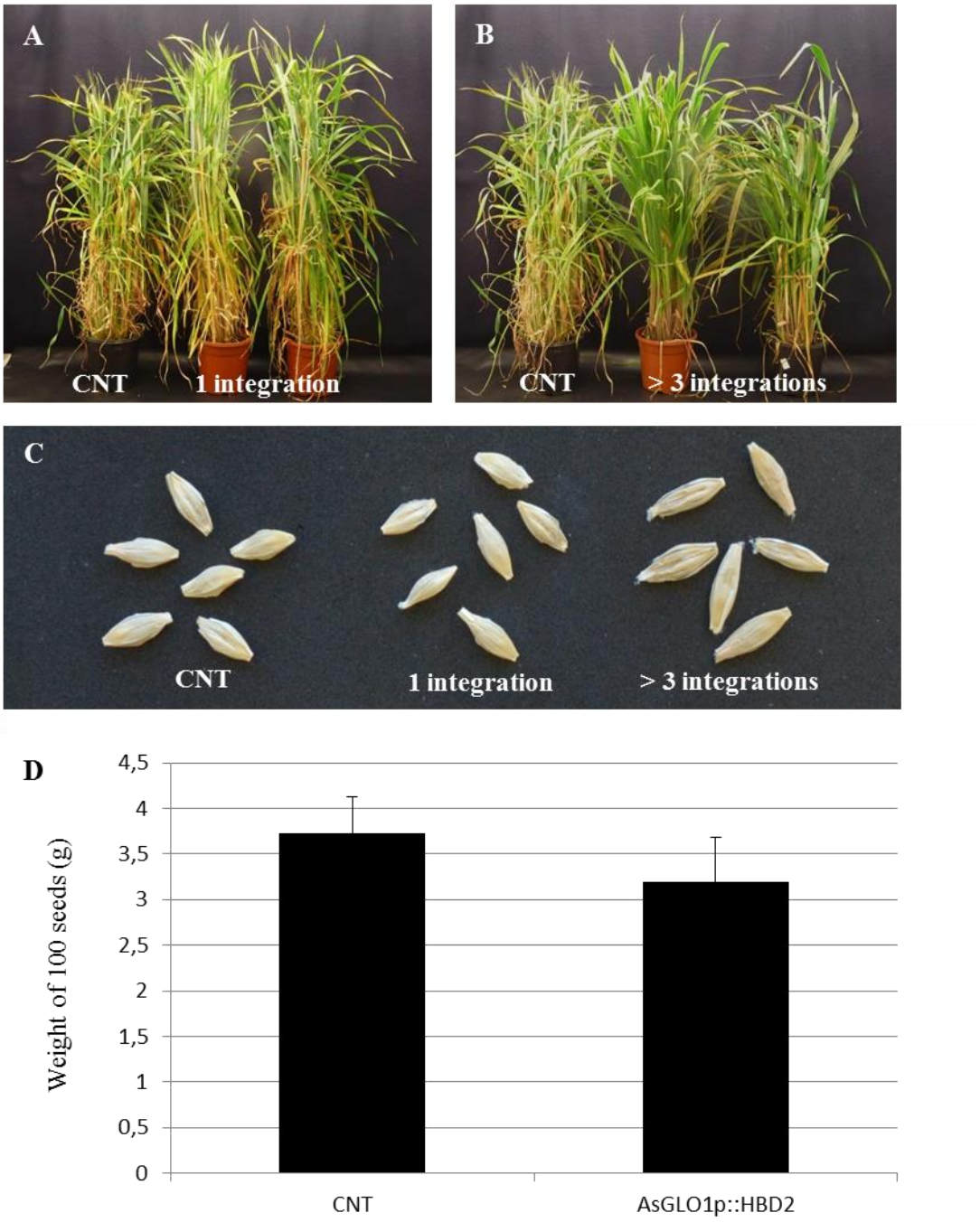

Figure 25. Comparison of phenotype of T1 transgenic barley plants expressing *rHBD2* under grain specific oat globulin gene promoter (*AsGLO1p*) to untransformed tissue culture regenerated barley plants (CNT). Representative photos of aerial part of plants harboring either 1 (A) or more than 3 integrations of transgene (B), structural characteristic of grains (C), and average weights of 100 mature grains of transgenic barley compared to CNT plants are indicated (D). Error bars represent the standard deviation of 3 independent lines per transformation event and at least 3 plants per line.

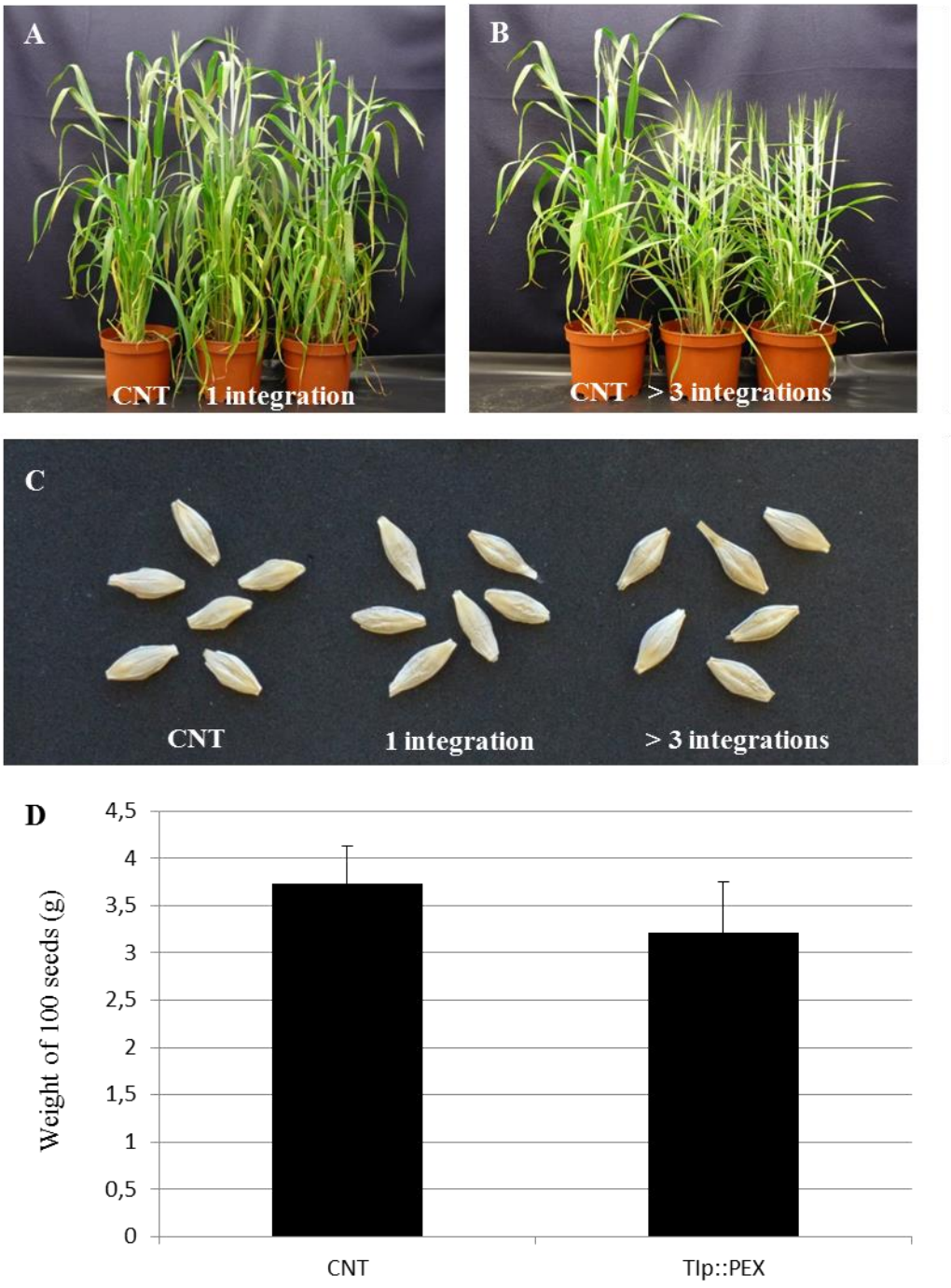

Figure 26. Comparison of phenotype of T1 transgenic barley plants expressing *rPEX* under grain specific trypsin inhibitor gene promoter (*TIp*) to untransformed tissue culture regenerated barley plants (CNT). Representative photos of aerial part of plants harboring either 1 (A) or more than 3 integrations of transgene (B), structural characteristic of grains (C), and average weights of 100 mature grains of transgenic barley compared to CNT plants are indicated (D). Error bars represent the standard deviation of 3 independent lines per transformation event and at least 3 plants per line.

3.8 Molecular analysis of regenerated barley plants at the genomic level

Based on PCR analysis results, from 5 to 37 independently transformed T0 barley plants for each of the DNA constructs shown in Figures 7C, 7D, 8A and 8B were selected. The transgenes were stably integrated into the barley genome and inherited (Fig. 27). 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 studies.

The transgene copy number was determined by segregation analysis (see Chapter 2.9) showing that less than 50% of the transformants were single copy T-DNA insertion lines with segregation ratio being 3:1. Furthermore, genomic DNA of randomly selected T0 lines was also subjected to Southern blotting using *hpt* gene-specific DIG-labelled DNA hybridization probes. The success of probe synthesis was always checked on agarose gel stained with ethidium bromide (Figure 28). As expected, obtained results were in concordance, as plants of 3:1 Mendelian segregation were confirmed to be a single copy T-DNA insertion lines using Southern blot (Figure 29), suggesting that both of the experimental approaches are applicable for this type of analysis.

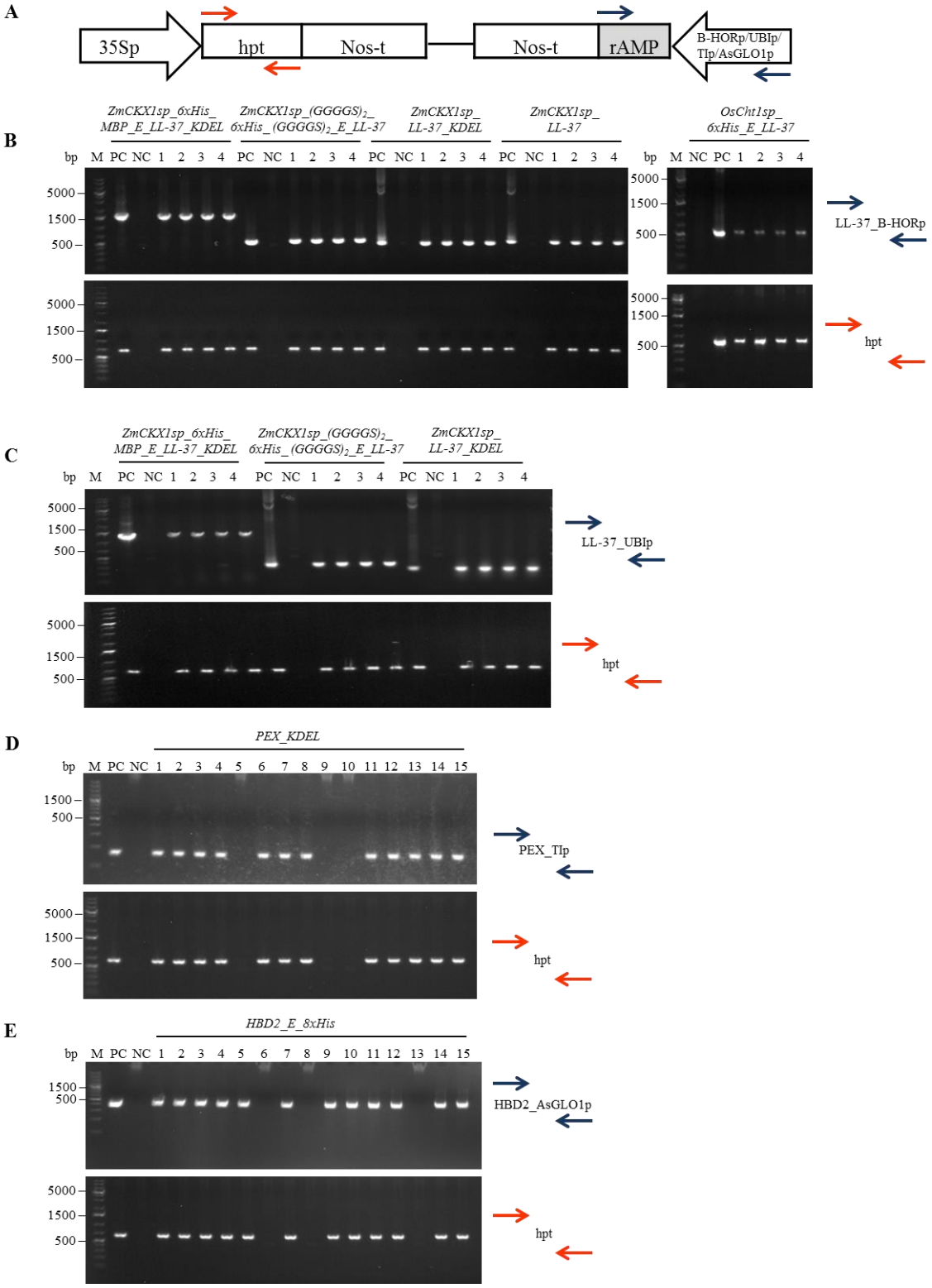

Figure 27. A representative figure showing detection of specific transgenes in T1 progeny barley lines transformed with the studied constructs. Approximate positions of the gene-specific oligonucleotides used for the PCR analysis in the DNA sequence are indicated by red and blue arrows in panel (A). Detection of transgenes in the lines expressing chimeric *LL-37* under the control of either the barley B1 hordein gene promoter (*B-HORp*) and the maize ubiquitin promoter (*UBIp*) is shown in (B) and (C), respectively. Results from analysis of the lines expressing recombinant pexiganan gene

(*PEX*) under the control of the trypsin inhibitor promoter (*TIp*) are shown in (D) and those from the lines expressing human beta-defensin 2 (*HBD2*) under the control of the oat globulin promoter (*AsGLO1p*) in panel (E). Amplification of the *hpt* gene fragment is shown in lower panels of B, C, D and E (expected amplicon size is 649 bp for all constructs used). Detection of the B1 hordein promoter sequence and the *LL-37* gene is shown in the upper panel. Expected fragment sizes (in bp) were as follows: *ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL* - 1681; *ZmCKX1sp_(GGGGS)2_6xHis_(GGGGS)2_E_LL-37* - 592; *ZmCKX1sp_LL-37_KDEL* -499; *ZmCKX1sp_LL-37* - 499; *OsCht1sp_6xHis_E_LL-37* – 538 (B). Detection of the maize ubiquitin promoter sequence and the *LL-37* gene is shown in the upper panel. Expected fragment sizes were as follows: *ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL* - 1379; *ZmCKX1sp_(GGGGS)2_6xHis_(GGGGS)2_E_LL-37* - 290; *ZmCKX1sp_LL-37_KDEL* – 197 (C). Detection of the trypsin inhibitor promoter sequence and the *PEX* gene is shown in the upper panel (173 bp) (D). Detection of the oat globulin promoter sequence and the *HBD2* gene is shown in the upper panel (482 bp) (E). M, DNA size marker 1 kb Plus; PC, positive control of the PCR reaction (pBRACT derived plasmids used for the barley transformation); NC, negative control of the PCR reaction (genomic DNA from control non-transgenic lines); 1 - 15, T1 barley lines.

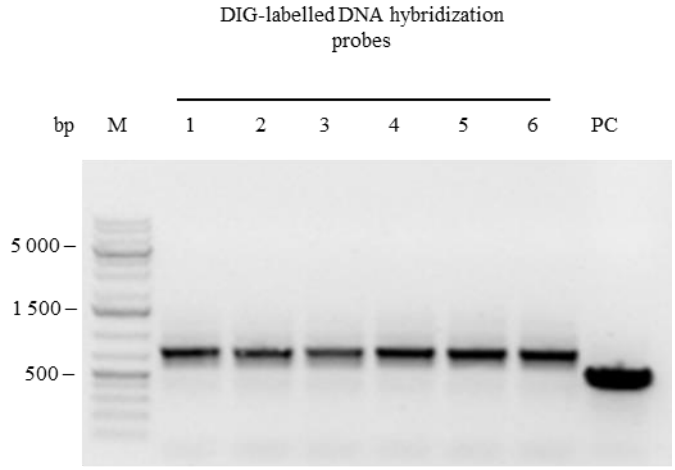

Figure 28. Representative results from evaluation of probe labelling efficiency. M, DNA size marker 1 kbp plus; 1 - 6, *hpt* gene-specific DIG-labelled DNA hybridization probes; PC, unlabeled positive control of the probe synthesis reaction (there were no DIG-dUTP in the reaction mixture). Expected amplicon size of the PC is 557 bp.

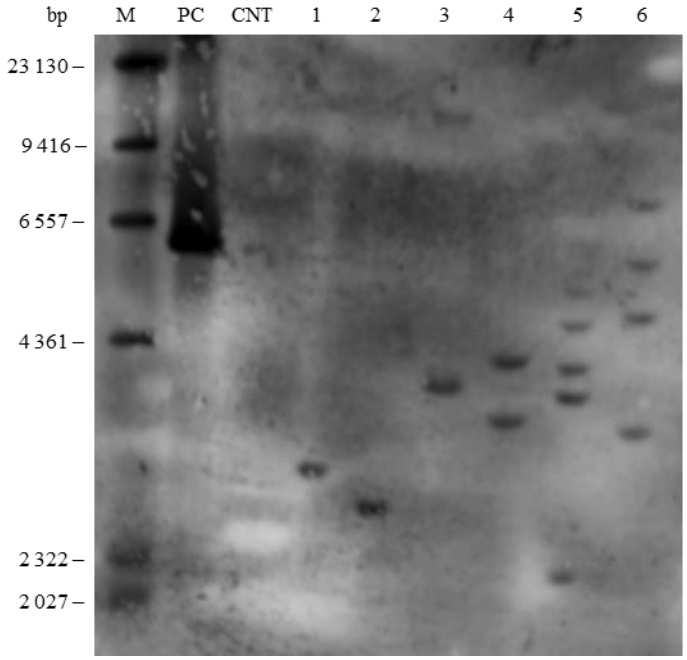

Figure 29. Representative results of Southern blot analysis of T-DNA transformants. *Xho*I digested DNA hybridized with a *hpt* probe was used. M, DNA size marker digoxigenin-labeled 0.12-23.1 kbp; PC, positive control (pBRACT derived plasmids used for the barley transformation); CNT, genomic DNA from control non-transgenic tissue culture regenerated barley plant; 1 - 6, gDNA from 6 independently transformed T0 transgenic lines with construct *ZmCKX1sp_(GGGGS)$_2$_6xHis_(GGGGS)$_2$_E_LL-37*. Note that most of the lines contain multiple copies of the T-DNA insertion.

3.9 Molecular analysis of regenerated barley plants on RNA level

First, to check wheter the transgene copy number is proportional to the gene expression level, c DNA isolates from late milk endosperm grains of selected T0 plants expressing *ZmCKX1sp_(GGGGS)$_2$_6xHis_(GGGGS)$_2$_E_LL-37* gene under *B-HORp* were analysed by means of qPCR assay. As depicted on Figure 30, no clear correlation between transgene expression on RNA level and transgene copy number was observed, as some of the multiple copy gene insertion lines showed one of the weakest and other ones the strongest expression.

Next, to see whether all *rLL-37* variants were successfully transcribed in roots, leaves and grains of the studied transgenic barley lines (Fig. 7CD), a set of randomly chosen T1 plants of *B-HORp* and *UBIp* lines was subjected to RT-PCR analysis using

primers specific to the coding regions of individual transgenes (Tab. 8). As shown in Figure 31, the *rLL-37* gene transcripts in the barley *B-HORp* lines were detected only in grains, but not in roots or leaves. Opposite to that, the *UBIp* lines showed the presence presence of *LL-37* amplicons in all analysed tissues. Hence, the functionality of the used promoters, as well as the transgenic expression of *rLL-37* genes, was clearly confirmed.

Analogously, the relative levels of transgene transcripts in selected T1 *PEX* and T2 *HBD2* expressing lines were determined, not only to confirm functionality of the expression vectors under study, but also to gain deeper insight into *TIp* and *AsGLO1p* activities. To do so, qPCR analysis was performed as described in Chapter 2.12. First, standard curves were run to calculate the efficiency of PEX (90.4%) and HBD2 (103%) primers. This was followed by determining the gene expression levels by qPCR assay and corrected by an efficiency factor. As depicted in Figure 32, all of the analysed lines showed highest product accumulation in seeds compared to roots or leaves. Expression controlled by *AsGLO1p* resulted in up to 157 000 times increase in levels of transgenic mRNA in soft dough endosperm grains (BBCH 85) compared to leaves. Contrary, the level of transgenic mRNA in soft dough endosperm grains of *rPEX* expressing lines was increased only up to 500 times compared to the leaves that showed weak non-specific expression. Beside that, transgenic RNA was also detected in the roots of all GMO lines analysed. However, there was much bigger difference between RNA levels of *rHBD2* in grains compared to roots, than in case of *rPEX* expressing lines (Figure 29). Hence, the *AsGLO1p* seems to be more appropriate for biotechnology applications using barley seeds as biofactories than the *TIp*, as it drives much stronger and target more effectively the expression of desired gene into this organ.

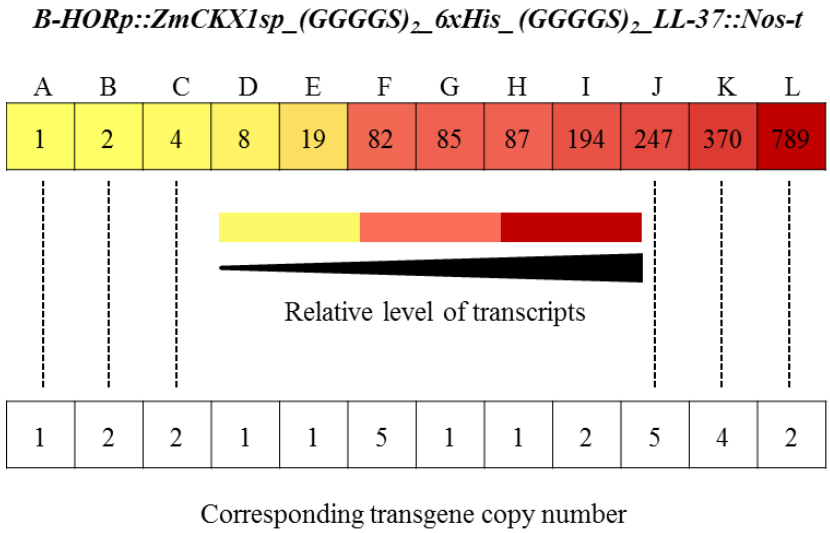

Figure 30: Transgene expression levels and transgene copy number of selected *rLL-37* barley lines. The amount of transgenic RNA was determined in the late milk endosperm grains (BBCH 77) of selected T0 lines (lines A – L) by qPCR assay. Corresponding transgene copy number was estimated by Southern hybridization analysis using *Xho*I digested barley genomic DNA that was hybridized with the *hpt* gene probe.

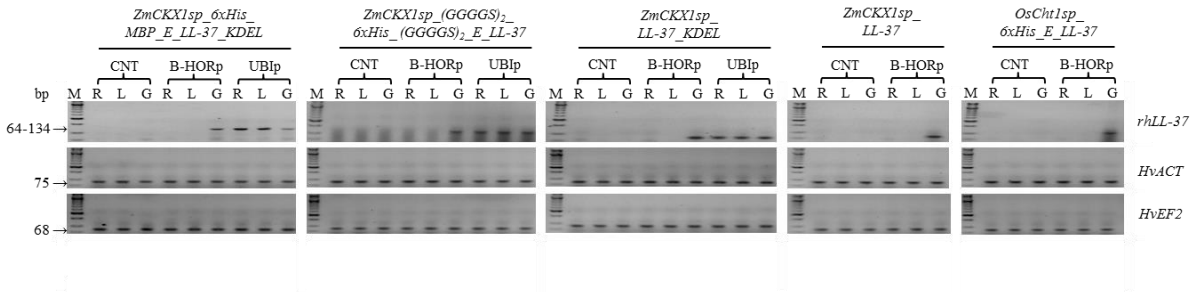

Figure 31. RT-PCR analysis of T1 transgenic barley lines expressing *rLL-37*. Barley actin (*HvACT*) together with the elongation factor 2 (*HvEF2*) genes served as loading controls. Expression was analysed in roots (R), leaves (L), and late milk (BBCH77) grains (G). CNT, cDNA of control nontransgenic *in vitro* regenerated plant; *B-HORp*, transgenic lines expressing *rLL-37* under grain specific B1 hordein gene promoter; *UBIp*, transgenic lines expressing *rLL-37* under maize ubiquitin gene promoter; M, 50 bp DNA size marker. Sequences of individual primers and expected amplicon sizes are summarized in Table 8.

A

	GRAINS			ROOTS	LEAVES
	BBCH 73	BBCH 85	BBCH 87	BBCH 73	BBCH 73
PEX_1	69 ± 8	14 ± 3	9 ± 2	8 ± 2	1
PEX_2	80 ± 19	484 ± 131	178 ± 20	7 ± 1	1

B

	GRAINS			ROOTS	LEAVES
	BBCH 73	BBCH 85	BBCH 87	BBCH 73	BBCH 73
HBD_1	50 235 ± 9 042	131 660 ± 30 281	120 460 ± 27 705	48 ± 19	1
HBD_2	124 647 ± 19 943	157 345 ± 36 189	93 298 ± 12 129	461 ± 51	1

Figure 32. Relative quantification of the pexiganan antimicrobial peptide (*PEX*, A) or the human beta-defensin 2 (*HBD2*, B) gene expression in various tissues of transgenic barley lines transformed with either pBRACT209/*TIp::PEX_KDEL::Nos-t* (A) or pBRACT209/*AsGLO1p::8xHis_E_HBD2::Nos-t* (B) expression cassette. Expression levels of target genes were quantified relative to basal transgene expression level detected in leaves of the same plants, which was set to 1. Experiment was always done with 4 to 6 different biological replicates for each independent transgenic line and each cDNA sample was analysed in 3 technical replicates. The DataAssist^{TM} software was used to evaluate the data using the ΔΔCt method corrected by an efficiency factor. Mean values with standard deviations (±SD) are shown. HBD2_1 and HBD2_2: two independent barley T2 transgenic lines expressing *HBD2* transgene; PEX_1 and PEX_2: two independent barley T1 transgenic lines expressing *PEX* transgene. BBCH 73, 85 and 87 represent early milk, soft dough and hard dough phenological development stages of barley used for this analysis.

3.10 Detection of rAMPs in barley grain sections by immunolabeling

To test the presence of rAMPs in transgenic barley lines and obtain more detailed information about their distribution/accumulation patterns, sectioned mature barley seeds were subjected to immunolabelling with specific antibodies as described in Chapter 2.13.

The presence of recombinant pexiganan and human beta-defensin 2 in confirmatory *in situ* immunolabeling tests resulted in the blue-violet coloration of endosperm of transgenic barley lines (Figure 33). *PEX* expressing lines showed non-specific staining of embryos, whereas no such coloration was observed for *HBD2* expressing lines. Differences may be attributed to different specificities of the antibodies used for the analysis.

Reagarding the visualisation of rLL-37 in barley grains, 2 various techniques were used, namely the fluorescence and the colorimetric detection. With the use of Alexa Fluor 488 probe it was possible to observe some local signals of emitting green light in *rLL-37* expressing plants (Figure 34), however the colorimetric method was shown to be more sensitive (Figure 35), hence was used for analysis of all transgenic barley lines expressing any variant of the *rLL-37* gene. All transgenic barley lines showed purple staining of endosperm reflecting the presence of rLL-37 peptide, while the endosperm of control lines remained unstained,. Much greater intensity in staining was achieved in the presence of the endoplasmic reticulum (ER) retention signal, the KDEL sequence at C-terminus. Staining in the grain sections that expressed the peptide under the control of *B-HOR* promoter was more intense than in the case of *UBI* promoter (Fig. 35A). Furthermore, as demonstrated in Figure 35B, accumulation pattern of rLL-37 was stably inherited over at least 3 successive generations.

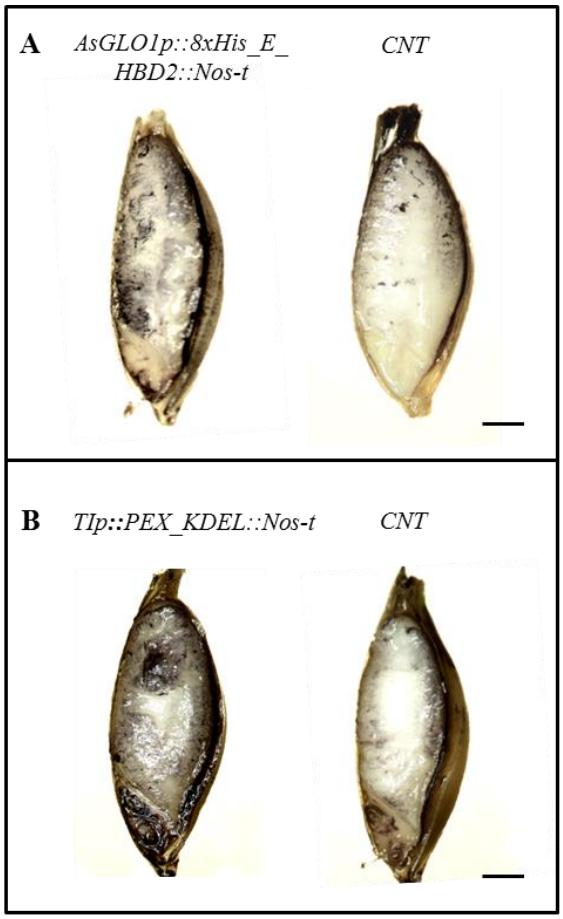

Figure 33. Representative figures of colorimetric detection of recombinant human beta-defensin 2 (HBD2, A) and pexiganan (PEX, B) antimicrobial peptide in desiccated seeds (BBCH 99) of T1 barley lines carrying the indicated transgenes. CNT, control non-transgenic tissue culture regenerated plant. Scale bars correspond to 1 mm.

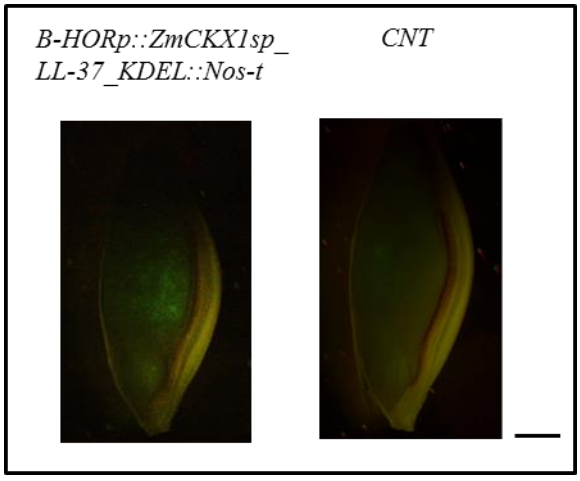

Figure 34: Immunolabeling of recombinant LL-37 (rLL-37) in desiccated seeds (BBCH 99) of T2 homozygous barley lines carrying the indicated transgene with the fluorescence probe Alexa Fluor 488. CNT, control non-transgenic tissue culture regenerated plant. Scale bar corresponds to 1 mm.

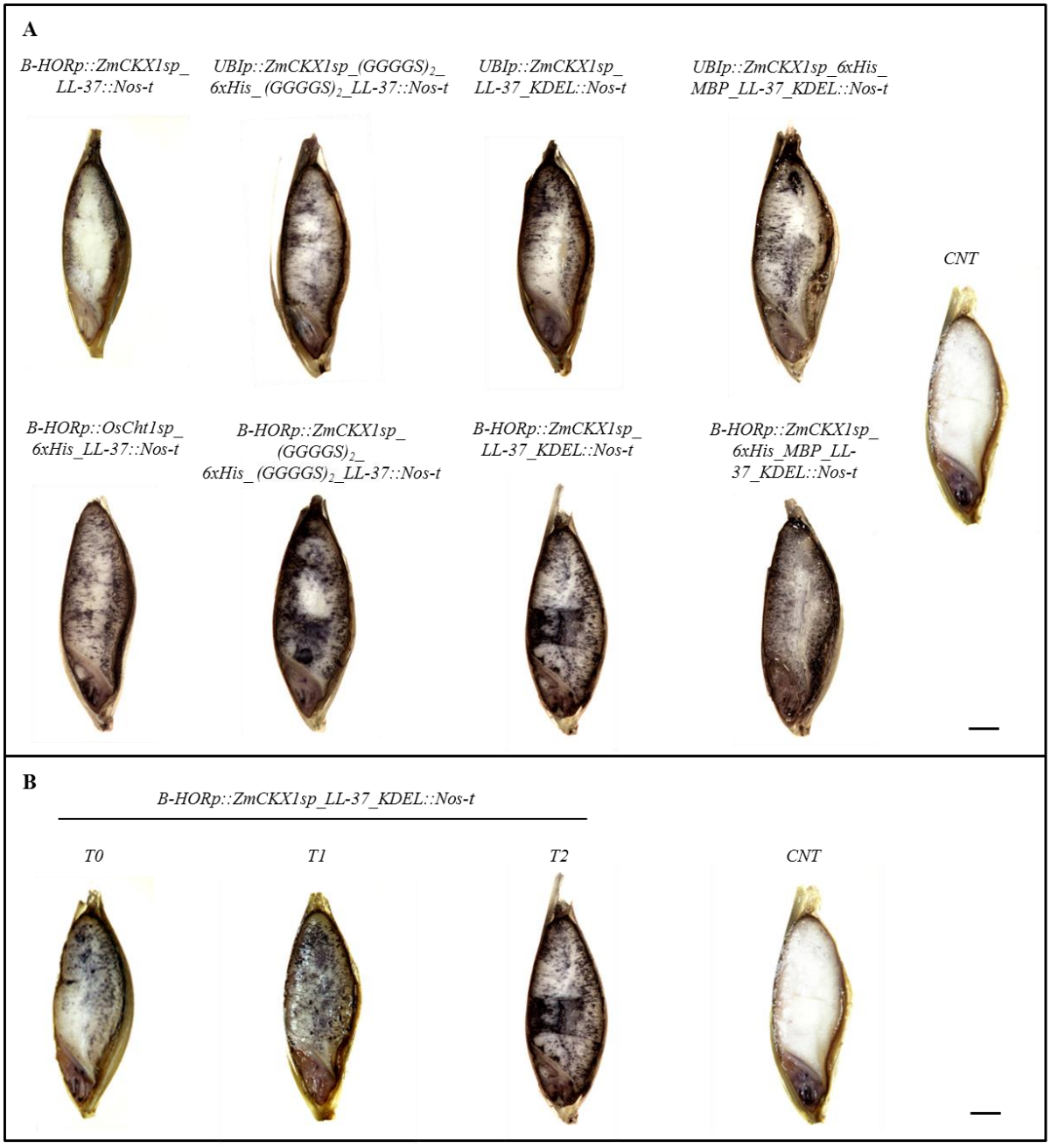

Figure 35: Representative figures of colorimetric detection of different variants of recombinant LL-37 (rLL-37) peptide in desiccated seeds (BBCH 99) of T2 barley lines carrying the indicated transgenes (A) and confirmation of stable integration and translation of integrated transgenes through three successive generations (T0, T1, T2; B). CNT, control non-transgenic tissue culture regenerated plant. Scale bars correspond to 1 mm.

3.11 Detection of rAMPs in protein extracts by Western blot

First, to investigate whether the LL-37, HBD2 and PEX are thermostable or heat labile, various amounts of synthetic commercially produced peptides were boiled either at 100 °C for 5 min or at 65 °C for 20 min, which was followed by Western blot analysis. All of the 3 AMPs tested were heat-stable, as neither degradation nor decrease in band intensity was observed (Figure 36). Hence, thermal heating of the analysed protein extracts should not lead to degradation of the peptides of interest.

Next, protein extracts from chosen extraction and purification steps described in Chapters 2.14 and 2.15 were subjected to Western blot analysis to see whether the transgene transcripts were properly translated in individual barley tissues. Unfortunately, none of the extract prepared from *rPEX* and *rHBD2* expressing lines, purified or not, as well as the protein bodies' enriched fractions gave positive result when analysed by Western blot (data not shown). This is in contrary to the findings obtained from immunolabeling analysis, that gave clear evidence about the presence of rPEX and rHBD2 AMPs in transgenic barley grains. Differences might be attributed to lower sensitivityof Western blot analysis where significantly higher content of the target peptide would be required to produce a detectable signal.

Whereas no specific signals were detected in crude protein extracts from leaves, roots and grains of the transgenic lines expressing rLL-37, Western blot analysis revealed the presence of rLL-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 (Fig. 37). When purifying rLL-37 peptides containing 6xHis and MBP epitope tags, the elimination of contaminating proteins by affinity chromatography greatly enriched the content of the corresponding rLL-37 peptide. Furthermore, when the epitope tags used for purification were cleaved off by enterokinase, the released rLL-37 peptides well matched the expected size (Fig. 37A). Purifications of ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL protein on either Co^{2+}-IDA-agarose or amylose affinity column provided comparable results. Non-tagged variants were not recovered from crude extracts in any case. Chimeric rLL-37 peptides extended with endoplasmic reticulum (ER) entry signal sequence ZmCKX1sp at *N*-terminus and *C*-terminal KDEL tetrapeptide ER retention sequence were successfully recovered

from protein bodies' enriched fraction (Fig. 37AB) and accordingly no product was extracted from protein bodies when lacking the KDEL sequence (data not shown). As shown in Table 10, content and stability of rLL-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 *B-HORp* provided much higher levels of rLL-37 peptide in grains than the expression driven by *UBIp*. Although analysis of *UBIp* lines provided clear evidence of the presence of rLL-37 peptide also in roots and leaves of transgenic plants (Fig. 37B), 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 rLL-37 peptide were obtained when produced as a fusion with ZmCKX1sp on *N*-terminus and *C*-terminal KDEL sequence (Table 10). 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 Western blot 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 10). 6xHis tag (Co^{2+}-IDA) purified protein fractions prepared from barley lines expressing *B-HORp*::*ZmCKX1sp_(GGGGS)$_2$_6xHis_(GGGGS)$_2$_LL-37* analysed by MALDI-TOF-MS showed a peptide of monoisotopic mass of 7161 Da, which was absent in the samples from control plants (analysis performed by prof. Mgr. Marek Šebela, Ph.D., Faculty of Science, Palacký University Olomouc). The detected mass exactly corresponds to that of the rLL-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.

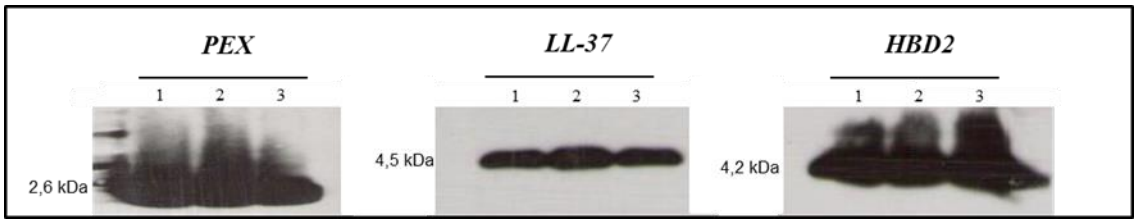

Figure 36: Demostration of thermal stability of indicated synthetic antimicrobial peptides by Western blot analysis. Amounts of 200 ng of PEX, 10 ng of LL-37, or 150 ng of HBD2 were mixed with loading buffer and reducing agent, which was followed by boiling at 70 °C for 10 min (lane 1). Alternatively, the samples were boiled either at 100 °C for 5 min (lane 2) or at 65 °C for 20 min (lane 3) prior to mixing with loading buffer and reducing agent and their subsequent boiling at 70 °C for 10 min. For details see Chapter 2.16.

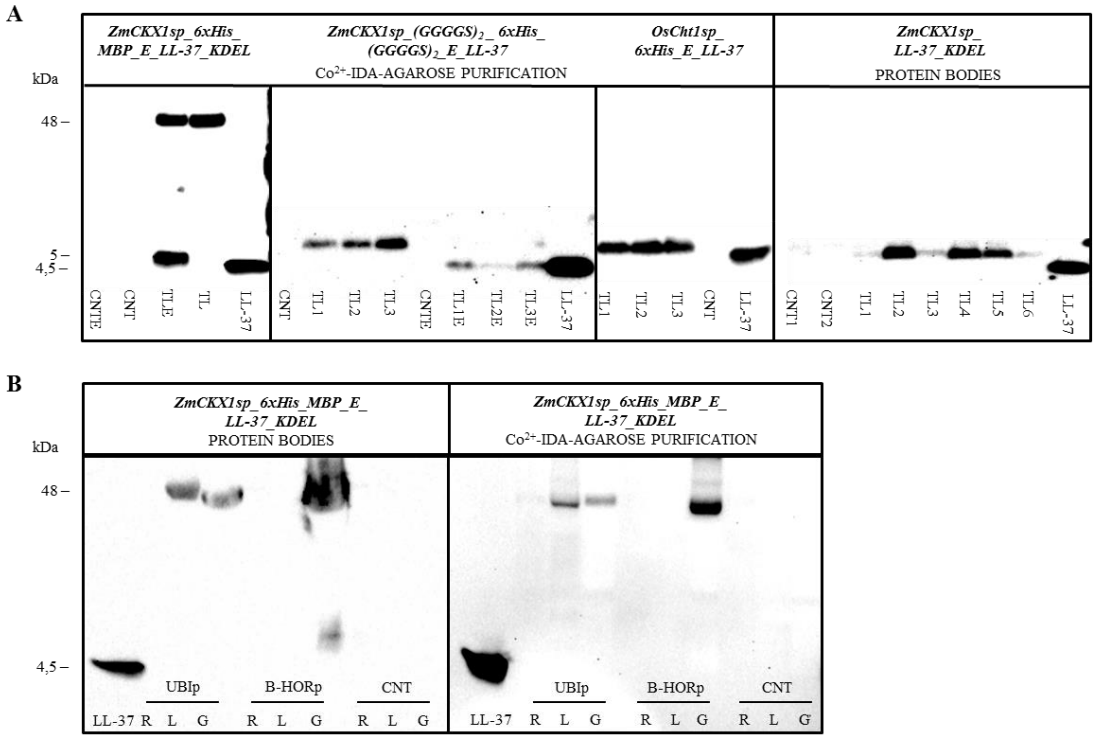

Figure 37. Western blot analysis of recombinant human LL-37 (rLL-37) in protein extracts of several T1 transgenic lines (T) and control non transgenic tissue culture regenerated plants (CNT). (A) Detection of rLL-37 in protein bodies or purified protein fractions from late milk endosperm seeds (BBCH 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 marked as (CNE) and (TLnE), respectively; 7 ng of synthetic LL-37 served as positive control. (B) Detection of rLL-37 in purified protein fractions prepared from roots (R), leaves (L) and late milk endosperm grains (G) of transgenic lines expressing the indicated transgenes under grain specific promoter of the barley B1 hordein gene (*B-HORp*), or the maize ubiquitin gene promoter (*UBIp*); 7 ng of synthetic LL-37 served as positive control.

Table 10. Accumulation levels of LL-37 in grains of T1 generation of transgenic barley lines as estimated by Western blot analysis of Co^{2+}-IDA purified protein extracts and protein body enriched fractions. BBCH scale 77 and 99 correspond to late milk endosperm seeds and desiccated seeds, respectively. *B-HORp*, transgenic lines expressing *rLL-37* under grain specific B1 hordein gene promoter; *UBIp*, transgenic lines expressing *rLL-37* under maize ubiquitin gene promoter. Displayed are the mean accumulation values with standard deviations of estimated amounts of rLL-37 peptide from 3 independent lines per transformation event and at least 3 plants per line (with the exception of *B-HORp::ZmCKX1sp_LL-37_KDEL*, where only 1 line was analysed). X, transgenic lines were not prepared; N.D. product not determined.

***rLL-37* gene**	**Co^{2+}-IDA-agarose purification** (μg of rLL-37 per kg of grains)				**Isolation of protein bodies** (μg of rLL-37 per kg of grains)			
	Late milk endosperm (BBCH 77)		Desiccated grain (BBCH99)		Late milk endosperm (BBCH 77)		Desiccated grain (BBCH 99)	
	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

3.12 Examination of antimicrobial activity of recombinant human LL-37 products

Since the antimicrobial activity of the LL-37 peptide has been documented elsewhere (Dürr *et al.*, 2006), only a simple test was conducted whether the rLL-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 (Gallo *et al.*, 2010). As shown in Fig. 38, 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 *B-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_E_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_$(GGGGS)_2$_E_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_E_LL-37* transgene, which peptide product is 6xHis_E_LL-37, resulted in 40% bacterial growth inhibition. To sum up, the rLL-37 peptide produced in barley plants including short tag elongated versions on either N- or C- termini are biologically active.

Protein extracts of barley *rPEX* and *rHBD2* expressing lines were not subjected to analysis of antimicrobial activity due to low concentration of AMPs in the protein extracts prepared (based on Western blot analysis results).

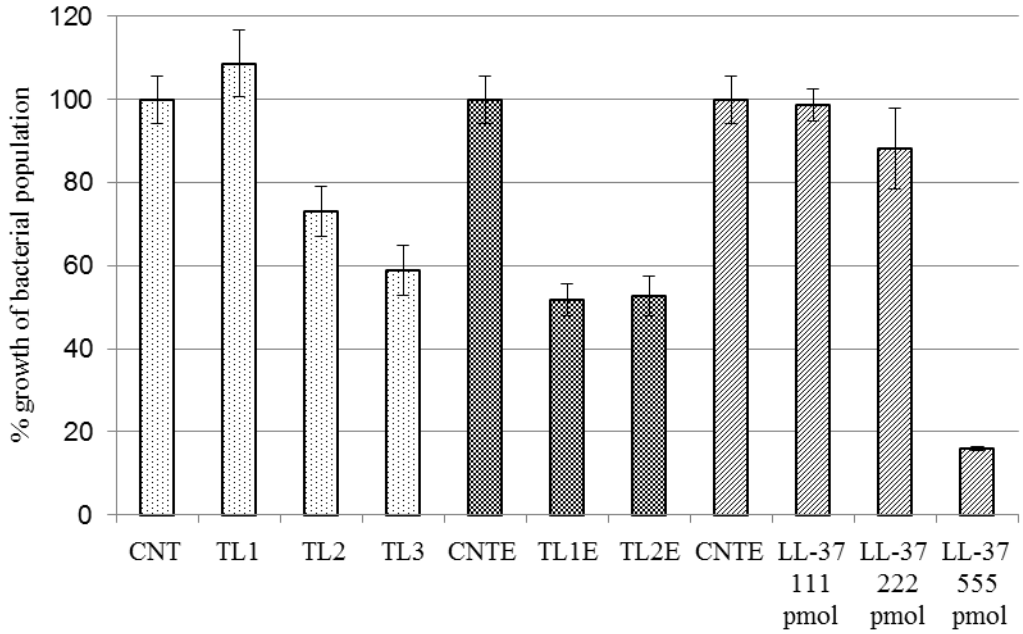

Figure 38. *In vitro* antibacterial activity of the synthetic LL-37 peptide and purified proteins prepared from late milk endosperm grains (BBCH 77) of T2 generation of transgenic lines. The biological activity was tested against *E. coli* TOP10, which have been mixed with 10 µL of Co^{2+}-IDA agarose purified fraction containing between 1 to 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. CNT, purified protein extracts from control non-transgenic tissue culture regenerated plants; TL1, purified protein extracts from lines expressing *ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL* transgene; TL2, *ZmCKX1sp_$(GGGGS)_2$_6xHis_$(GGGGS)_2$_E_LL-37* transgene; TL3, *OsCht1sp_6xHis_E_LL-37* transgene. CNTE, enterokinase digested purified protein extracts from control plants; TL1E, enterokinase digested purified protein extracts from lines expressing *ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL* transgene; TL2E, *ZmCKX1sp_$(GGGGS)_2$_6xHis_$(GGGGS)_2$_E_LL-37* transgene. Error bars represent standard error of results of two independent experiments.

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4 Discussion

Although AMP production in plants represents very promising strategy for modern applications in healthcare and agriculture, there is a challenging path to meet the demands for commercialization of these systems. Many factors must be considered including compatibility of the product with the expression system, plant-specific post-translational modifications, product yield, storage conditions, purification strategy and others (Broz *et al.*, 2013). Most processes in plant-based AMP production should largely follow classical regulatory requirements already established for non-plant produced recombinant pharmaceuticals as well as regulatory requirements applied on GM plants. There are also several unique challenges regarding AMP production in plants, which arise from the simple fact that certain plant production systems might include use of food crops and involve production of pharmaceutical peptides in open field. All pharmaceutical AMPs produced in plants should be assessed favourably on their safety as well as efficacy by the legislative agencies. Related with GM plant risk assessment and regulatory framework, there are considerable differences between Europe and the United States (Sparrow *et al.*, 2013). For example in the US, there are three coordinated agencies responsible for regulation of all biotechnological products, namely the Food and Drug Administration (FDA), the Environmental Protection Agency (EPA) and the US Department of Agriculture (USDA). Each of these institutional bodies has its own regulatory scope (Shama and Peterson, 2004). In Europe, risk assessment related with GM plants is carried out by the European Food Safety Authority (EFSA). Since open field *in planta* molecular pharming is associated with potential risk of pollen escape and contamination of the food supply (Shama and Peterson, 2004), there are strict regulations on physical isolation of GM crops from non-transgenic plants (Elbehri, 2005). As the isolation requirement largely influences the cost in PMF, barley as a self-pollinating cereal crop with its heavy pollen was selected as a host plant, since it minimize the risk of gene flow due to its physiological properties. The isolation distances of barley are maximally several tens of metres, which is in contrast to at least one mile isolation distance for maize with its wind-borne, relatively light pollen (Elbehri, 2005).

Economic feasibility of AMP production in plants is mainly dependent on development of a proper business strategy to match the anticipated benefit with biopharming; i.e., reduced cost of goods. One of the basic and most difficult practical considerations is the choice of final pharmaceutical product itself. As AMPs are considered as prospective therapeutic agents, it is no wonder, that they are being extensively studied, as reflected by the number of scientific articles published in recent years. There were 9 172 new publications on this topic during the year 2018, which corresponds to about 25 articles per each day (https://www.ncbi.nlm.nih.gov/pubmed/, downloaded 22.5.2019). In the present study, 3 antimicrobial peptides were selected out of the pool of existing AMPs based on intensive search in literature and subsequently expressed in transgenic barley and eventually also in tobacco leaves. Two of the main attributes for their selection taken under consideration were as follows: structure of the target peptide should not be cyclic due to possible difficulties connected to its proper folding, and the selected peptide should be applicable in commercial sector, e.g. cosmetic or pharmaceutical industry. Below there will be first discussed possible applications of the selected AMPs in modern medicine.

First of the promising candidates fulfilling the requirements stated above include the LL-37. As already stated in introduction to this thesis, not only is the peptide an efficient antibacterial, antiviral and antifungal killer, but also mediates additional immune reactions such as wound healing, inflammatory responses or inhibition of tumorigenesis. Some of the examples of its potential practical application include the use of LL-37 as adjuvant in antibiotic-adjuvant pairing. Synergistic effects of LL-37 and antibiotics cause exert of therapeutic and bactericidal activity against target bacteria, including some of the MDR (multi-drug resistant) bacteria (e.g. *Klebsiella pneumonia*, *Pseudomonas aeruginosa, Acinetobacter baumannii*) that are of medical importance (Lin *et al.*, 2015; Nuding *et al.*, 2014). Quite recently, it has been shown that LL-37 might represent an Alzheimer's disease therapy candidate, as it is able to specifically bind to Aβ (a pathophysiological protein of Alzheimer's disease), and hence hinders its pathological accumulation (De Lorenzi *et al.*, 2017). Furthermore, LL-37 is being investigated in phase I clinical trial for its ability to induce anti-tumor immunity of melanoma patients with dermal metastases when applied in form of intra-tumoral injections (https://clinicaltrials.gov/ct2/show/NCT02225366, downloaded 28.5.2019). LL-37 might also be used in form of local application to the skin for the treatment of patients suffering from chronig leg ulcers, as described in a study

by Grönberg and co-workers (2014). In general, local application of antimicrobial peptides in form of creams, shampoos, lotions, and wound dressings seems to be the most advantageous over other possible forms of their administration, as they are susceptible to proteolysis by host peptidases, which may lead to loss or at least reduction of their effectivity (Rahnamaeian & Vilcinskas, 2015). Furthermore, when applied locally, AMPs might be delivered in higher concentrations to the affected area, as their absorption to the bloodstream and the associated side effects would be limited. Next AMP that was selected for its heterologous expression in barley includes pexiganan. In particular, its administration could be useful to overcome antibiotic resistance, as it possess broad antimicrobial spectra including MDR strains of bacteria while it retains low toxicity to mammalian cells. Pexiganan has undergone clinical trials for treatment of various more or less serious skin diseases. These incude diabetic foot ulcers, that might be treated in form of pexiganan acetate (1%), which would be locally applied as a cream to the affected area. However, although it was well tolerated by patients, the AMP has not been approved for marketing so far due to the fact that it is not more efficient than drugs already available on the market (Lipsky *et al.*, 2008). Besides that, pexiganan also entered clinical trials for the treatment of children with impetigo (https://adisinsight.springer.com/drugs/800002904, downloaded 3.6.2019). In addition, pexiganan could also serve in combination with approved β-lactam or glycylcycline antibiotics for the treatment of sepsis, as it possess synergistic activity with them (Cirioni *et al.*, 2018; Giacometti *et al.*, 2005). Finally, the third peptide selected for its expression in transgenic barley plants includes the human beta-defensin 2. Studies on antiviral activities of HBD2 showed that the peptide is active against severe human viral pathogens including human immunodeficiency virus, influenza A virus, and respiratory syncytial virus. These advantages could be taken for practical application of HBD2 to combat these viral infections. Moreover, human defensins may be also used as therapeutic adjuvants or vaccine carriers, as their presence leads to the production of specific cytokines that are responsible for better recovery from infections plus they also markedly enhance the efficacy of therapeutic antibodies (Park *et al.*, 2018). Furthermore, it was demonstrated that β-defensins 2 might be utilized for second generation cancer vaccines, due to their ability to chemotax immature dendritic cells (Biragyn, 2005). Another proposed utilization of human defensins, when supplied in a controlled manner,

includes the “defensine vaccine” concept to protect human body against viral infections, which would be particularly useful during the flu season (Park *et al.*, 2018).

Although a number of advantageous features make not only LL-37, pexiganan and HBD2, but also other AMPs attractive as potential therapeutic compounds, their commercial use is primarily hampered due to high production costs. For example, the price of chemically synthesized LL-37 ranges from thousands to tens of thousands of Czech crowns (CZK) per mg depending on purity. The price of synthetic HBD2 is even one order of magnitude higher than the price of LL-37, meaning that 1 mg of the peptide costs from about tens of thousands to hundreds of thousands of CZK. Finally, the price of synthetic pexiganan is in scope of thousands of CZK per mg. Plant based producion of AMPs provides tremendous benefits over other production systems, especially in terms of production costs. However, certain technical limitations still remain. These are in general connected to the quantity and quality of the produced peptide, that reflects not only the amount of expressed transgenic RNA, but also the efficiacy of its translation, stability and biological activity of the produced peptide and its recovery. Additionaly, the expressed AMP might exhibit a certain level of phytotoxicity, that may cause abnormal undesirable plant phenotype. Accordingly, many parameters needf to be optimized on both RNA and protein levels to boost the yield of the final product. As its has shown to be problematic to express peptides of less than 50 amino acids in cells of cereal plants, possibly due to the silencing mechanisms or rapid degradation of the peptide (Takagi *et al.*, 2008; Takagi *et al.*, 2010; Yasuda *et al.*, 2005), several plant expression vectors for the production of LL-37 fused to various functional protein or peptide domains were designed and screened for evaluation of yield of expression before they were employed for preparation of stably transgenic barley plants. For the screening purposes served agroinfiltration of tobacco leaves. Results of this analysis clearly showed that N- terminal fusion of LL-37 to sorting signal sequence seems to be essential to be the transgene expressible and subsequently detectable on protein level, probably due to fast degradation of those products lacking it. Furthermore, it has been shown previously that an addition of KDEL tetrapeptide is essential to target *in planta* produced recombinant proteins to membrane-bound organelles, either the so-called protein bodies (PB-I) (Bundó *et al.*, 2014; Company *et al.*, 2014), or to protein storage vacuoles (PB-II) (Acralis *et al.*, 2004) that are responsible for so-called bioencapsulation of the product.

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 (Stöger *et al.*, 2005). Effect of the ER retention sequence on the higher productivity of recombinant LL-37 has been confirmed in transient tobacco assay where 3-fold higher accumulation was observed in the case of construct bearing the KDEL sequence. Accordingly, all of the *LL-37* gene variants designed for the expression in transgenic barley included N- and eventually also C- terminal KDEL targeting signal to direct the AMP accumulation to certain subcellular compartments. 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 (Bell *et al.*, 2013). 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.

Selection of promoter sequence is no less important for proper transgene expression in transgenic plant, as it holds the key to match the requirements for high expression levels. Accordingly, it was necessary to select promoters suitable for *AMPs* expression in barley. In numerous studies, expression of *AMP* was driven by the constitutive *UBIp*. Advantages of constitutive *AMP* expression in barley may lay in high product yield, as a recombinant product could be theoretically recovered from all of its tissues. On the other hand, ubiquitous expression and subsequent accumulation of an AMP might negatively affect biological functions in a host plant as stated in introduction to this thesis. With this respect, organ-specific regulation of gene expression can help reduce this risk. Regarding barley, grains represent the most suitable target tissue for recombinant protein accumulation, as their natural properties should enable not only robust protein accumulation, but also its long-term storage and simple downstream processing. Since an endosperm forms approximately three quarters of a barley grain, thus represent its largest morphological part, it is advantageous to direct the expression to this tissue. In addition, the barley grain endosperm is used by ORF genetics to produce biorisk-free growth factors (epidermal and stem cell growth factors) and cytokines for use in medical research, skin care and stem cell technology, that are available under the trademarks ISOkine ™ and Bioeffect ™ (http://www.orfgenetics.com/, downloaded 20/06/2019). The company built their

large greenhouses in Iceland, where most of the primary energy supply is derived from renewable or cheap sources such as geothermal energy, hence price of energy in Iceland is among the cheapest in the world. In scope of this study, several candidate promoters driving endosperm-preferred expression were selected from a barley genetic background using Genevestigator screening tool. As a next step, strength of the expression driven by the candidates during development of a barley grain was compared, as application of strong promoters able to drive expression in later phases of endosperm tissue development seems to be beneficial for the purpose of plant molecular farming. The promoter of the barley B1 hordein gene followed by the trypsin inhibitor gene promoter showed the best results in this respect, thus both of them were employed in *AMPs* expression strategies. Since the functionality of the *B-HORp* has been verified previously by generation of transgenic lines overexpressing the barley *CKX9* gene (Holásková, 2012), plus its properties were deeply characterized in scope of this thesis using the *CKX9* overexpressors, it was incorporated in most of the vectors for grain-specific accumulation of AMPs.

Results of this thesis clearly showed, that neither ubiquitous nor grain specific accumulation of LL-37 resulted in abnormal growth characteristics and morphology of the transgenic barley lines and the same was true for single copy T-DNA lines expressing *rPEX* gene. Contrary to that, single copy *rHBD2* expressing lines showed extended phenotype that might be attributed to the type of AMP expressed and its properties that negatively affected barley plant growth, as this phenomenon was observed in all of the 3 independent transgenic T1 and T2 transgenic lines examined. Regarding analysis of multiple copies T-DNA insertion lines expressing either the pexiganan or the human beta-defensin 2, there were observed significant changes in the plant phenotype as well as in a rate of the *rHBD2* expressing plant growth. These deviations might be attributed to 1) a properties of the transgene product expressed, 2) a somaclonal variation (Larkin & Scowcroft, 2006) that is caused by structural changes and changes in the number of chromosomes which can occur in plants regenerated from cell cultures (Vyroubalová *et al.*, 2011), 3) a site of integration, as integration of a transgene into a host genome is more or less random process, hence such integration may induce changes in the plant phenotype. However, it is necessary to emphasize, that there was in both of the cases (meaning *rPEX* and *rHBD2* expressing lines) analysed only one independent transgenic line

harbouring the insert in multiple copies and hence no conclusions can be drawn due to lack of additional information.

As the *in situ* immunolabeling analysis confirmed, although at various extend, the presence of all of the recombinant products under study in mature barley grains, next goal was their isolation. Unfortunately, neither the Western blot analysis of protein extracts of *rPEX* nor *rHBD2* expressing lines gave positive result. This failure might attributed to the absence of peptide localization sequence at the N-terminus, which was probably reflected in low stability of the product and subsequently its very low level in mature barley grains. These results are in concordance to those obtained from agroinfiltration of tobacco leaf tissues with constructs for transient expression of human cathelicidin variants lacking N-terminal translocation signal. Besides, negative results may be ascribed to inappropriate extraction techniques used that resulted in low extraction efficiency. In contrast to that, 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. The amount of produced rLL-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 (Bundó *et al.*, 2014). Cabanos *et al.* (2013) 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 (Lee *et al.*, 2011; Zeitler *et al.*, 2013). 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.

Analysing 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. Although the MBP fusion was expected to boost the amount of recombinant protein product in all of the stages of barley grain development, total accumulation of MBP fused to LL-37 was lower than this of free peptide in milky endosperm, but fused peptide was slightly more abundant in desiccated grain than the non-fused rLL-37 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 rLL-37 peptide. In both cases, a purified peptide fraction was obtained. Higher yields of the peptide, however, were achieved by isolating the ER-derived protein bodies (into which LL-37 was deposited when expressed with the secretion signal peptide and the C-terminal tetrapeptide KDEL) by simple gradient centrifugation in sucrose solution according to Bundó *et al.* (2014) and thereby, purification time and costs were significantly reduced compared to chromatographic methods. Comparison of product amount of rLL-37 obtained using isolation of protein bodies versus affinity purification has shown that the loss of recovery yields of rLL-37 fused to MBP represented about 85% in late milk endosperm grains and about 99% in desiccated grains when using chromatography column separation. Hence, targeting into storage organelles seems to be much more beneficial for subsequent industrial or pharmaceutical large scale production of AMPs. In addition, a study by Takagi and co-workers (2010) revealed, that cereal seed ER-derived protein body may represent an effective tool for oral delivery of peptide and protein therapeutics. Authors of this study demonstrated, that a tolerogen for the control of pollen allergy when localized in rice seed derived PB-I showed increased protection from the enzymatic digestion in simulated gastric fluid.

The LL-37 peptide's antibacterial activity is conformation-dependent. It can adopt either disordered or helical structure which represents its biologically active form. Transition from disordered structure to alpha-helix positively correlates with the antimicrobial activity of the peptide. At micromolar concentration, the LL-37

peptide exists in water in a disordered structure. Formation of alpha-helix is dependent on LL-37 peptide concentration (the higher the concentration, the greater extent of helical conformation), on the pH value and on the presence of anions such as HCO_3^-, CO_3^{2-} , or SO_4^{2-} (Gallo *et al.*, 2010; Johansson *et al.*, 1998). Accordingly, all of the purified rLL-37 either cleaved by enterokinase or not, were buffer exchanged for NH_4HCO_3 in order to test their ability to inhibit bacterial growth. The LL-37 exhibited biological activity against *E. coli* TOP 10 cells either after cleavage of the tag in the case of MBP or even in a fusion with a smaller 6xHis tag or KDEL tetrapeptide. These results are in concordance with a study by Mičúchová (2017), who showed similar results in terms of retaining biological activity of LL-37 fused to C-terminal KDEL extension. In addition, Coca and co-workers (2006) and also Bundó and co-workes (2014) have demostrated, that rice plant produced cecropin A peptide designed for retention in endoplasmic reticulum exhibited antifungal and antibacterial activity, respectively. On the other hand, the antimicrobial activity of the LL_KDEL product was unsuccessfully tested by Janechová (2018), who subjected the peptide for the antibacterial activity assay against a gram-positive bacterium *Micrococcus luteus*, even though that the group by Kim *et al.* (2009) observed clear growth inhibition zone against *M. luteus* when placing recombinant hCAP18/LL37 produced in its active form by *Pichia pastoris* on top of the test media poured by tested bacterial culture (Kim *et al.*, 2009). Observed variations in rLL-37 activity might be attributed to different methods used for functional assessment of this peptide, as it has been shown previously, that antimicrobial efficiency of AMPs strongly depends on assay parameters including the applied medium in which the microbes are treated (Farkas *et al.*, 2018). Together these data indicate, that although rLL-37 antimicrobial properties were confirmed using *E. coli* TOP10 cells, there is no guarantee that *in planta* produced recombinant LL-37 that has been shown to be effective against one bacterial strain using one test assay will also be active against another pathogen using different assay indicating that it is necessary to optimize the antimicrobial activity assay for each case.

5 Conclusions

In conclusion, human cathelicidin LL-37, human beta-defensin 2 and *Xenopus laevis* derived pexiganan were selected from diverse natural and synthetic peptides with antimicrobial activities for production in barley, as they may represent valuable products for innovative applications in medical or cosmetic industry. Although heterologous expression of AMPs using plant-based systems is regarded as a key to the bottleneck for their large-scale cost-efficient production, many technical limitations that must be surmounted still remain. To respond accurately to this challenge, several modifications of *LL-37* gene were designed and evaluated for yield using agroinfiltration of tobacco leaf tissues. Based on results obtained from the screening technology used, stable transgenic barley lines expressing various codon-optimized *AMP* fusion genes either under constitutive or selected grain specific promoter were generated and analysed. Most of the transgenic plants showed similar growth rate dynamics and morphological characteristics as the control tissue culture regenerated lines. Immunolabeling using specific antibodies confirmed the accumulation of individual AMPs, although to various extents, in barley grain endosperm, and stable expression and storage of the product at room temperature over several years in ceed cellular background was confirmed by analysis of 3 successive generations of B-HORp::ZmCKX1sp_LL-37_KDEL::Nos-t lines. It was demonstrated that human cathelicidin can be produced by molecular farming in barley either as peptide *sensu stricto* just with the ER retention tetrapeptide or in the fusion with MBP that increases its stability in desiccated grain and cleavage of the LL-37 fusion protein using enterokinase results in efficient removal of the tags from recombinant products containing DDDDK pentapeptide. It was also shown, that the grain-specific expression provides higher product yields in grains than the constitutive one and the largest accumulation occurs in the stage of milky endosperm. The C- terminal KDEL extension in combinantion with N-terminal signal peptide sequence of *Zea mays* cytokinin oxidase/dehydrogenase 1 resulted in accumulation of the product in ER- derived protein bodies (PB-I), as confirmed by subcellular fractionalization. Furthermore, isolation of the recombinant LL-37 by preparation of a protein body enriched fraction resulted in higher product recovery compared to the protein isolation using chromatographic methods. This attribute along with the fact that a presence of the KDEL sequence should not have negative impact

on LL-37 antimicrobial properties, as demonstrated by antibacterial activity assay using *E. coli* TOP 10 cell, and together with the fact that bioencapsulation should be resposible for at least partial protecion of recombinanat proteins or peptides from enzymatic digestion by host proteases, make this technology ideal for production of peptide therapeutics. To sum up, results of this work show that biologically active AMPs can be produced in barley using various viable approaches, which might be in future applicable for their commercial production (Fig. 39).

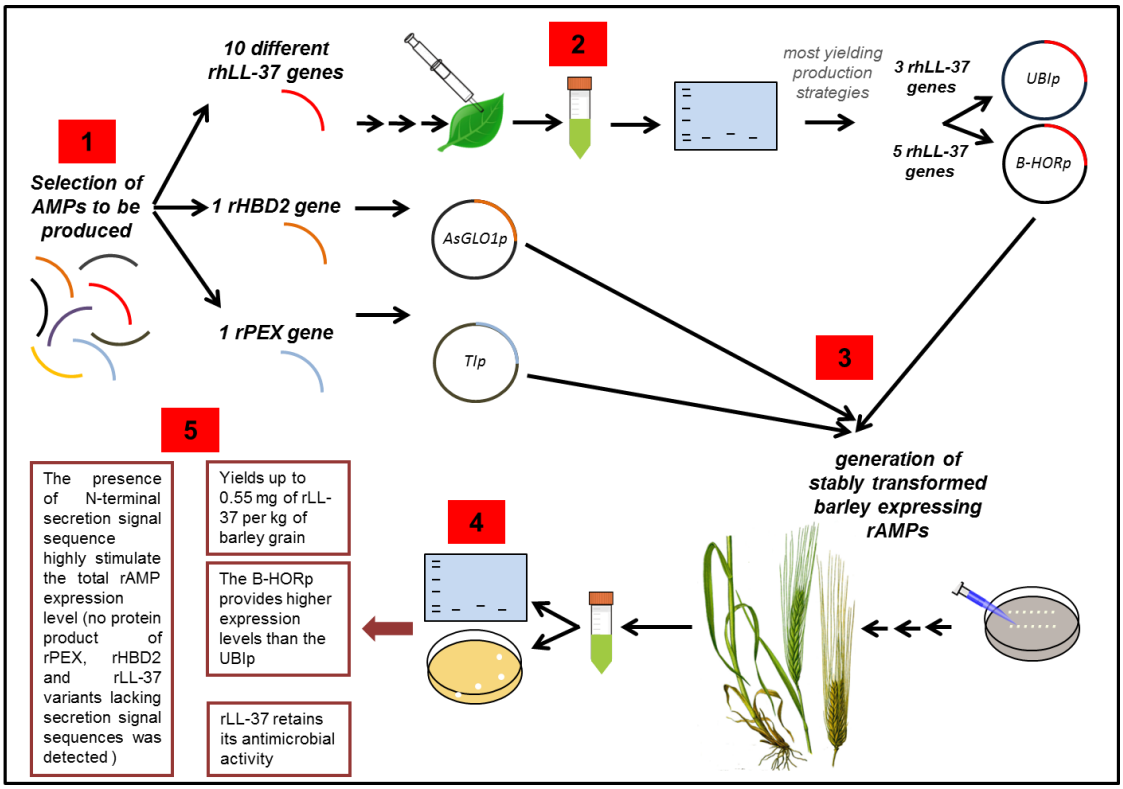

Figure 39. Flow diagram highlighting the key points of this work. (1) Some of the antimicrobial peptides (AMPs) that might be applicable in therapeutic or cosmetic industry were selected from large varieties of AMPs. (2) Effects of different fusion partners on AMP production using transient expression in tobacco leaves was assessed. (3) Selected chimeric genes were cloned under chosen promoters and used for the generation of transgenic barley lines expressing *AMPs*. (4) The effect of different expression strategies on accumulation levels and antimicrobial activity was assessed. (5) Results of this work provide important data for plant molecular farming with low molecular weight peptides.

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7 Abbreviations

A	aleurone
ACT	actin
ACHE-I-7.1	peptide mimetic of aldicarb
AMP	antimicrobial peptide
AMY	α-amylase
AsGLO1	*Avena sativa* globulin 1
BASI	barley α-amylase/subtilisin inhibitor
BBBI	Bowman-Birk type trypsin inhibitor
B-HOR	barley B1 hordein
BP1	barley peroxidase 1
BPH	barley peroxidase homolog
BSA	bovine serum albumin
BSSP	barley seed specific peroxidase 1
BY-2	tobacco Bright Yellow-2 cells
CAMP	cathelicidine anti-microbial peptide
CaMV	cauliflower mosaic virus
cDNA	complementary deoxyribonucleic acid
CFU	colony forming units
CI	chymotrypsin inhibitor
CKX/CKO	cytokinin oxidase/dehydrogenase
CM	chloroform methanol extracted proteins
CNT	control
COL1A1	collagen α-1 chain
CRISPR	clustered regularly interspaced short palindromic repeats
CSFV	classical swine fever virus
CYC	cyclophilin
DAPI	4′,6-diamidino-2-phenylindole
D-HOR	barley D hordein

DIG	digoxigenin
DNA	deoxyribonucleic acid
DTT	dithiotreitol
E	enterokinase recognition sequence
ECL	enhanced chemiluminescent substrate
EDTA	ethylenediaminetetraacetic acid
EF	elongation factor
EGTA	ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N′*,*N′*-tetraacetic acid
EM	embryo
EN	endosperm
EPA	Environmental Protection Agency
ER	endoplasmic reticulum
ETEC	F4-positive enterotoxigenic *Escherichia coli*
F	forward
FAEG	immunogenic fimbral adhesin
FDA	Food and Drug Administration
gDNA	genomic deoxyribonucleic acid
GM	genetically modified
GRAS	generally regarded as safe
HBD2	human beta-defensin 2
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hinb	hordoindoline b
His	histidine
HIV	human immunodeficiency virus
HMW	high-molecular-weight
hNP	human neutrophil peptide
HOR2-4	barley hordein B-1
HOR3-1	barley D hordein
hpt	hygromycin phosphotransferase

Hv	*Hordeum vulgare*
IDA	iminodiacetic acid
iP9G	N^6-isopentenyladenine-9-glucoside
LB	left border
LB media	Luria-Bertani media
LEV-I-7.1	levamisole-mimetic synthetic peptide
LL-37	the only human antimicrobial peptide of cathelicidin family
LPS	lipopolysacharides
LTP	lipid transfer proteins
M	DNA size marker
MALDI-TOF	matrix-assisted laser desorption/ionization with time-of-flight detection mass spectrometry
mas	*mannopine synthase*
MBP	maltose-binding protein
mRNA	messenger ribonucleic acid
MS	mass spectrometry
Nbe	*Nicotiana benthamiana*
ND	non-determined
NMR	nuclear magnetic resonance
Nos-t	the terminator of nopaline synthase gene of *Agrobacterium tumefaciens*
OsCht1	*Oryza sativa* chitinase 1 signal peptide
OsGLUB-1	*Oryza sativa* endosperm-specific glutelin B-1
p	promoter
PB-I	protein bodies
PB-II	protein storage vacuoles
PC	positive control
PCR	polymerase chain reaction
PEX	pexiganan
pHvSUMO	putative barley small ubiquitin like modifier
PMF	plant molecular farming

pro	protease recognition sequence
PVDF	polyvinylidene difluoride
qPCR	real-time quantitative polymerase chain reaction
r	recombinant
R	reverse
RB	right border
RNA	ribonucleic acid
RsAFP	*Raphanus sativus* antifungal protein
RT-PCR	real-time reverse transcription polymerase chain reaction
S	SYBR Green chemistry
SD	standard deviation
SERPINS	serin protease inhibitors
sp	signal peptide
SUMO	small ubiquitin like modifier
T	TaqMan chemistry
TCA	trichloroacetic acid
T-DNA	transfer DNA
TI	trypsin inhibitor
TL	transgenic line
TMV	tobacco mosaic virus
TRIS	tris(hydroxymethyl)aminomethane
TSP	total soluble protein
UBI	ubiquitin
USDA	the United States Department of Agriculture
X	factor Xa recognition sequence
ZmCKX1sp	*Zea mays N*-terminal secretion signal sequence of cytokinin dehydrogenase 1
ZmUBI	*Zea mays* ubiquitin-1
35Sp	35S promoter from the cauliflower mosaic virus

8 Curriculum vitae

Personal data

Name and surname: Edita Holásková
Place and date of birth: Kroměříž, 20th March 1988
Nationality: Czech
Contact: edita.holaskova@upol.cz

Education

2012 – present
Palacký University in Olomouc
Ph.D. study in Biochemistry

- Research project: Generation and analysis of new transgenic barley lines expressing recombinant antimicrobial peptides.

2010 – 2012
Palacký University in Olomouc
MSc. Biochemistry

- Research project: Preparation of transgenic barley with cytokinin altered level in grains.

2007 – 2010
Palacký University in Olomouc
BSc. Biochemistry

- Research project: Monitoring of the influence of purine derivates on root development of *Arabidopsis thaliana*.

Employment

2012 – present
Student research assistant
Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University in Olomouc

Research fellowships

July – August 2015
Biotechnology Research Center, Shanxi Academy of Agricultural Sciences, Taiyuan , China prof. Yi Sun

- The goal was to get practical experience in 2 various methods for genetic manipulation of maize, that were developed at the local research center.
- Oral presentation of results of my Ph.D. research project.

January – March 2014
Freie Universität, Berlin, Bundesrepublik Deutschland

- The goal was to analyse HIPP transformants of *Arabidopsis thaliana* at the protein level.
- Oral presentation of results of my Ph.D. research project.

Teaching

Academic year 2016/2017
Basic laboratory techniques for biochemists (KBC/LABT)

- 5 hours/week

Academic year 2015/2016
Basic laboratory techniques for biochemists (KBC/LABT)

- 5 hours/week

Advanced biochemical methods (KBC/BAM)

- Topic: Genetic modification of cereals

Academic year 2014/2015
Basic laboratory techniques for biochemists (KBC/LABT)

- 10 hours/week

Academic year 2013/2014
Basic laboratory techniques for biochemists (KBC/LABT)

- 5 hours/week

Summer biotechnology project 1 (KBC/LBP1)

- Student: Alžbeta Mičúchová, Topic: Gene cloning

Academic year 2012/2013
Basic laboratory techniques for biochemists (KBC/LABT)

- 5 hours/week

Supervisior of master student:	Alžbeta Mičúchová, defended in 2017
Supervisor of bachelor student:	Alžbeta Mičúchová, defended in 2015 Viktor Valtera, defended in 2016 Veronika Janechová, defended in 2016

Oral conference presentation

2017
Holásková E, Galuszka P, Frébort I. Transgenic barley as a bioreactor for the production of recombinant cationic α-helical antimicrobial peptides. *Green for Good IV*, Olomouc, Czech Republic.

Poster conference presentations

2015

Holásková E, Öz MT, Mičúchová A, Galuszka P. Plant-based production of antimicrobial peptides with therapeutic potential. *Green for Good III*, Olomouc, Czech Republic.

2014

Holásková E, Öz MT, Galuszka P, Frébort I. Molecular Farming: Plants as Vehicles for the Production of Antimicrobial Peptides. *12th PhD Student Conference of Plant Experimental Biology*, Olomouc, Czech Republic.

2014

Holásková E, Öz MT, Galuszka P, Frébort I. (2014): Molecular Farming: Plants as Vehicle for the Production of Antimicrobial Peptides. *The first Conference of the International Society for Plant Molecular Farming*, Berlin, Bundesrepublik Deutschland.

2013

Holásková E, Öz MT, Galuszka P, Frébort I. (2013): Biopharming as a tool for production of antimicrobial peptides. *2nd Conference of Cereal Biotechnology and Breeding*, Budapest, Hungary.

Publications

Holásková E, Galuszka P, Mičúchová A, Šebela M, Öz MT, Frébort I. 2018. Molecular farming in barley: development of a novel production platform to produce human antimicrobial peptide LL-37. *Biotechnology Journal* 13(6): e1700628.

Holásková E, Galuszka P, Frébort I, Öz MT. 2015. Antimicrobial peptide production and plant-based expression systems for medical and agricultural biotechnology. *Biotechnology Advances* 33, 1005-1023.

Mrízová K, Holásková E, Öz MT, Jiskrová E, Frébort I, Galuszka P. 2014. Transgenic barley: a prospective tool for biotechnology and agriculture. *Biotechnology Advances* 32, 137-147.

Fields of interest

Molecular biology methods:	PCR methods, cloning, Southern blot, Western blot, etc.
Biochemical methods:	recombinant protein expression, protein isolation and purification, electrophoretic methods, analysis of biological activity of recombinant products, etc.
Genetic modification of plants:	stable transformation of barley, infiltration of tobacco leaves

Awards

2013: Awarded by director of the CRH for excellence in category "Contracted research" for verification of production of antimicrobial peptides in the grain of transgenic barley.

Skills

Language:	English, German
Driving licence:	B
Computer skills:	MS Windows; MS Word; MS Excel; MS PowerPoint; Clone Manager; BioEdit; Primer Express; DataAssist; Picasa

Others

Member of the Academic Senate of the Faculty of Science of Palacký University Olomouc

9 Supporting informations

A) **LL-37** 38 AA

MLLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES

B) **ZmCKX1sp_LL-37** 55 AA

[MAVVYYLLLAGLIACSHA]**LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES**

C) **ZmCKX1sp_LL-37_KDEL** 59 AA

[MAVVYYLLLAGLIACSHA]**LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTESKDEL**

D) **6x His_MBP_E_LL-37** 431 AA

MHHHHHHKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEE
KFPQVAATGDGPDIIFWAHDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVR
YNGKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPALDKELKAKGKSALMFNLQ
EPYFTWPLIAADGGYAFKYENGKYDIKDVGVDNAGAKAGLTFLVDLIKNKHM
NADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSKVNYGVTVLPTFKGQPSKP
FVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVALKSYEEE
LAKDPRIAATMENAQKGEIMPNIPQMSAFWYAVRTAVINAASGRQTVDEALKD
AQTNSSSNNNNNNNNNNLG*DDDDK***LLGDFFRKSKEKIGKEFKRIVQRIKDFL**
RNLVPRTES

E) **ZmCKX1sp_6xHis_MBP_E_LL-37** 448 AA

[MAVVYYLLLAGLIACSHA]HHHHHHKIEEGKLVIWINGDKGYNGLAEVGKKF
EKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIFWAHDRFGGYAQSGLLAEITP
DKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPAL
DKELKAKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVGVDNAG
AKAGLTFLVDLIKNKHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSKV
NYGVTVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVN
KDKPLGAVALKSYEEELAKDPRIAATMENAQKGEIMPNIPQMSAFWYAVRTA
VINAASGRQTVDEALKDAQTNSSSNNNNNNNNNNLG*DDDDK***LLGDFFRKSK**
EKIGKEFKRIVQRIKDFLRNLVPRTES

F) **ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL** 452 AA

[MAVVYYLLLAGLIACSHA]HHHHHHKIEEGKLVIWINGDKGYNGLAEVGKKF
EKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIFWAHDRFGGYAQSGLLAEITP
DKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPAL
DKELKAKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVGVDNAG
AKAGLTFLVDLIKNKHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSKV
NYGVTVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVN
KDKPLGAVALKSYEEELAKDPRIAATMENAQKGEIMPNIPQMSAFWYAVRTA
VINAASGRQTVDEALKDAQTNSSSNNNNNNNNNNLG*DDDDK***LLGDFFRKSK**
EKIGKEFKRIVQRIKDFLRNLVPRTESKDEL

G) **6xHis_SUMOstar_LL-37** 145 AA

MGHHHHHHG<u>SLQDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTTP
LRRLMEAFAKRQGKEMDSLTFLYDGIEIQADQTPEDLDMEDNDIIEAHREQIGG</u>
LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES

H) **ZmCKX1sp_6xHis_SUMOstar_LL-37** 162 AA

[MAVVYYLLLAGLIACSHA]GHHHHHHGSLQDSEVNQEAKPEVKPEVKPETHIN
LKVSDGSSEIFFKIKKTTPLRRLMEAFAKRQGKEMDSLTFLYDGIEIQADQTPED
LDMEDNDIIEAHREQIGG**LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRT**
ES

I) **ZmCKX1sp_6xHis_pHvSUMO_E_LL-37** 161 AA

[MAVVYYLLLAGLIACSHA]HHHHHHSGVTADEDKKPAGDGGGAHINLKVKG
QDGNEVFFRIKRSTQLKKLMNAYCDRQSVDLNSIAFLFDGRRLRGEQTPDELE
MEEGDEIDAMLHQTGG*DDDDK***LLGDFFRKSKEKIGKEFKRIVQRIKDFLRN**
LVPRTES

J) **6x His_pHvSUMO_X_LL-37** 147 AA

MGHHHHHHGSLQGVTADEDKKPAGDGGGAHINLKVKGQDGNEVFFRIKRSTQ
LKKLMNAYCDRQSVDLNSIAFLFDGRRLRGEQTPDELEMEEGDEIDAMLHQTG
G*IEGR***LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES**

K) **ZmCKX1sp_$(GGGGS)_2$_6xHis_$(GGGGS)_2$_E_LL-37** 86 AA

[MAVVYYLLLAGLIACSHA]$(GGGGS)_2$HHHHHH$(GGGGS)_2$*DDDDK***LLGDFFRK**
SKEKIGKEFKRIVQRIKDFLRNLVPRTES

L) **OsCht1sp_6xHis_E_LL-37** 68 AA

[MRALAVVVVATAFAVVAVRG]HHHHHH*DDDDK***LLGDFFRKSKEKIGKEFK**
RIVQRIKDFLRNLVPRTES

M) **PEX_KDEL** 27 AA

MGIGKFLKKAKKFGKAFVKILKKKDEL

N) **8x His_HBD2** 53 AA

MHHHHHHHH*DDDDK***DPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCK KPN**

Figure S1. Amino acid sequences of the putative chimeric rLL-37 proteins expressed either in *Nicotiana benthamiana* leaves (A-J) or in barley (B, C, F, K, L), and putative chimeric pexiganan (M) and recombinant human beta-defensin 2 (N) expressed in transgenic barley. Sequences enclosed in square brackets correspond to the signal peptide sequence of *Zea mays* cytokinin oxidase/dehydrogenase 1 (ZmCKX1sp) or *Oryza sativa* chitinase 1 (OsCht1sp), bold sequences to the human LL-37 antimicrobial peptide/or pexiganan antimicrobial peptide/ or human beta-defensin 2 antimicrobial peptide with or without the KDEL extension, plain text sequences to purification tags (MBP tag and/or 6x His tag or 8x His tag); underlined sequences correspond to SUMO derived fusion proteins (SUMOstar or putative barley SUMO / pHvSUMO) and the sequence recognized by protease (enterokinase (E)/Factor Xa (X)) is written in italics.

A) **B-HORp::ZmCKX1sp_LL-37_KDEL::Nos-t**

Barley B1 hordein gene promoter: 13 bp – 562 bp

Kozak consensus sequence: 581 bp – 589 bp

ZmCKX1 signal peptide: 590 bp – 640 bp

LL-37: 641 bp – 751 bp

KDEL signal peptide: 752 bp – 763 bp

Stop codon: 764 bp – 766 bp

Nos terminator: 791 bp – 1074 bp

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        10        20        30        40        50        60        70        80        90       100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTTAAAGTCGACGTGCAGGTGTATGAGTCATTGTTATGATCTATAGGTGTCAGTTTATCTTATCATCTGGGTGATCAATACAGGCCCAGGTTTTATAAAA

       110       120       130       140       150       160       170       180       190       200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACCAGTCGAGTCGAGAAGAACCGTCCACATGTAAAGCTTTAACAACCCACACATTGATTGCAACTTAGTCCTACACAAGTTTTCCATTCTTGTTTCAGGC

       210       220       230       240       250       260       270       280       290       300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TAACAACCTATACAAGGTTCCAAAATCATGCAAAAGTGATGCTAGGTTGATAATGTGTGACATGTAAAGTGAATAAGGTGAGTCATGCATACCAAACCTC

       310       320       330       340       350       360       370       380       390       400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GGGATTTCTATACTTTGTGTATGATCATATGCACAACTAAAAGGCAACTTTGATTATCAATTGAAAAGTACCGCTTGTAGCTTGTGCAACCTAACACAAT

       410       420       430       440       450       460       470       480       490       500
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTCCAAAAATCCATTTGCAAAAGCATCCAAACACAATTGTTAAAGCTGTTCAAACAAACAAAGAAGAGATGAAGCCTGGCTACTATAAATAGGCAGGTAG

       510       520       530       540       550       560       570       580       590       600
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TATAGAGATCTACACAAGCACAAGCATCAAAACCAAGAAACACTAGTTAACACCAATCCACTGTCGACGGTACCGGATCCGCCACCATGGCCGTTGTTTA

       610       620       630       640       650       660       670       680       690       700
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTACCTCCTCCTCGCCGGCCTCATTGCCTGCTCTCACGCCCTCCTCGGCGATTTCTTCCGCAAGTCCAAGGAGAAGATCGGCAAGGAGTTCAAGAGGATC

       710       720       730       740       750       760       770       780       790       800
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTCCAGAGGATCAAGGACTTCCTCCGCAACCTCGTGCCAAGGACCGAGAGCAAGGACGAGCTTTGACTCGAGCCTTGGTCTAGAGAGCTCAGTCAAGCAG

       810       820       830       840       850       860       870       880       890       900
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACGTTAAGCATG

       910       920       930       940       950       960       970       980       990
1000
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATA

      1010      1020      1030      1040      1050      1060      1070      1080      1090
1100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCGACCGGCATGCAAGCTGATATGAGCTCGCATGCGCGGCCGCGATATC
```

B) **<u>UBIp::ZmCKX1sp_LL-37_KDEL::Nos-t</u>**

Maize ubiquitin promoter: 1 bp – 1987 bp

Kozak consensus sequence: 2056 bp – 2064 bp

ZmCKX1 signal peptide: 2065 bp – 2115 bp

LL-37: 2116 bp – 2226 bp

KDEL signal peptide: 2227 bp – 2238 bp

Stop codon: 2239 bp – 2241 bp

Nos terminator: 2335 bp – 2618 bp

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         10        20        30        40        50        60        70        80        90       100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTGCAGCGTGACCCGGTCGTGCCCCTCTCTAGAGATAATGAGCATTGCATGTCTAAGTTATAAAAAATTACCACATATTTTTTTTGTCACACTTGTTTGA

        110       120       130       140       150       160       170       180       190       200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AGTGCAGTTTATCTATCTTTATACATATATTTAAACTTTACTCTACGAATAATATAATCTATAGTACTACAATAATATCAGTGTTTTAGAGAATCATATA

        210       220       230       240       250       260       270       280       290       300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AATGAACAGTTAGACATGGTCTAAAGGACAATTGAGTATTTTGACAACAGGACTCTACAGTTTTATCTTTTTAGTGTGCATGTGTTCTCCTTTTTTTTTG

        310       320       330       340       350       360       370       380       390       400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CAAATAGCTTCACCTATATAATACTTCATCCATTTTATTAGTACATCCATTTAGGGTTTAGGGTTAATGGTTTTTATAGACTAATTTTTTTTAGTACATCT

        410       420       430       440       450       460       470       480       490       500
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATTTTATTCTATTTTAGCCTCTAAATTAAGAAAACTAAAACTCTATTTTAGTTTTTTTATTTAATAATTTAGATATAAAATAGAATAAAATAAAGTGACT

        510       520       530       540       550       560       570       580       590       600
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AAAAATTAAACAAATACCCTTTAAGAAATTAAAAAAACTAAGGAAACATTTTTCTTGTTTCGAGTAGATAATGCCAGCCTGTTAAACGCCGTCGACGAGT

        610       620       630       640       650       660       670       680       690       700
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTAACGGACACCAACCAGCGAACCAGCAGCGTCGCGTCGGGCCAAGCGAAGCAGACGGCACGGCATCTCTGTCGCTGCCTCTGGACCCCTCTCGAGAGTT

        710       720       730       740       750       760       770       780       790       800
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCGCTCCACCGTTGGACTTGCTCCGCTGTCGGCATCCAGAAATTGCGTGTCGGACGGCAGACGTGAGCCGGCACGGCAGGCGGCCTCCTCCTCCTCTCAC

        810       820       830       840       850       860       870       880       890       900
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GGCACCGGCAGCTACGGGGGATTCCTTTCCCACCGCTCCTTCGCTTTCCCTTCCTCGCCCGCCGTAATAAATAGACACCCCCTCCACACCCTCTTTCCCC

        910       920       930       940       950       960       970       980       990
1000
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AACCTCGTGTTGTTCGGAGCGCACACACACACAACCAGATCTCCCCCAAATCCACCCGTCGGCACCTCCGCTTCAAGGTACGCCGCTCGTCCTCCCCCCC

       1010      1020      1030      1040      1050      1060      1070      1080      1090
1100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCCCCCTCTCTACCTTCTCTAGATCGGCGTTCCGGTCCATGGTTAGGGCCCGGTAGTTCTACTTCTGTTCATGTTTGTGTTAGATCCGTGTTTGTGTTAG

       1110      1120      1130      1140      1150      1160      1170      1180      1190
1200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATCCGTGCTGCTAGCGTTCGTACACGGATGCGACCTGTACGTCAGACACGTTCTGATTGCTAACTTGCCAGTGTTTCTCTTTGGGGAATCCTGGGATGGC

       1210      1220      1230      1240      1250      1260      1270      1280      1290
1300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCTAGCCGTTCCGCAGACGGGATCGATTTCATGATTTTTTTTTGTTTCGTTGCATAGGGTTTGGTTTGCCCTTTTCCTTTATTTCAATATATGCCGTGCA

       1310      1320      1330      1340      1350      1360      1370      1380      1390
1400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTTGTTTGTCGGGTCATCTTTTCATGCTTTTTTTTGTCTTGGTTGTGATGATGTGGTCTGGTTGGGCGGTCGTTCTAGATCGGAGTAGAATTAATTCTGT

       1410      1420      1430      1440      1450      1460      1470      1480      1490
1500
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTCAAACTACCTGGTGGATTTATTAATTTTGGATCTGTATGTGTGTGCCATACATATTCATAGTTACGAATTGAAGATGATGGATGGAAATATCGATCTA
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        1510      1520      1530      1540      1550      1560      1570      1580      1590
1600
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GGATAGGTATACATGTTGATGCGGGTTTTACTGATGCATATACAGAGATGCTTTTTGTTCGCTTGGTTGTGATGATGTGGTGTGGTTGGGCGGTCGTTCA

        1610      1620      1630      1640      1650      1660      1670      1680      1690
1700
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTCGTTCTAGATCGGAGTAGAATACTGTTTCAAACTACCTGGTGTATTTATTAATTTTGGAACTGTATGTGTGTGTCATACATCTTCATAGTTACGAGTT

        1710      1720      1730      1740      1750      1760      1770      1780      1790
1800
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TAAGATGGATGGAAATATCGATCTAGGATAGGTATACATGTTGATGTGGGTTTTACTGATGCATATACATGATGGCATATGCAGCATCTATTCATATGCT

        1810      1820      1830      1840      1850      1860      1870      1880      1890
1900
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTAACCTTGAGTACCTATCTATTATAATAAACAAGTATGTTTTATAATTATTTTGATCTTGATATACTTGGATGATGGCATATGCAGCAGCTATATGTGG

        1910      1920      1930      1940      1950      1960      1970      1980      1990
2000
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATTTTTTTAGCCCTGCCTTCATACGCTATTTATTTGCTTGGTACTGTTTCTTTTGTCGATGCTCACCCTGTTGTTTGGTGTTACTTCGCCCATCACAAGT

        2010      2020      2030      2040      2050      2060      2070      2080      2090
2100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTGTACAAAAAAGCAGGCTGGCGCCGGAACCAATTCAGTCGACCGTACGGGATCCGCCACCATGGCCGTTGTTTACTACCTCCTCCTCGCCGGCCTCATT

        2110      2120      2130      2140      2150      2160      2170      2180      2190
2200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GCCTGCTCTCACGCCCTCCTCGGCGATTTCTTCCGCAAGTCCAAGGAGAAGATCGGCAAGGAGTTCAAGAGGATCGTCCAGAGGATCAAGGACTTCCTCC

        2210      2220      2230      2240      2250      2260      2270      2280      2290
2300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GCAACCTCGTGCCAAGGACCGAGAGCAAGGACGAGCTTTGACTCGAGGAATTCGCGGCCGCACTCGAGATATCTAGACCCAGCTTTCTTGTACAAAGTGG

        2310      2320      2330      2340      2350      2360      2370      2380      2390
2400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TGATGGGGGATCCACTAGTTCTAGAATTCGATTGAGTCAAGCAGGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCT

        2410      2420      2430      2440      2450      2460      2470      2480      2490
2500
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TGCGATGATTATCATATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAGA

        2510      2520      2530      2540      2550      2560      2570      2580      2590
2600
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCGAC

        2610
....|....|....|...
CGGCATGCAAGCTGATAT
```

C) **B-HORp::ZmCKX1sp_$(GGGGS)_2$_6xHis_$(GGGGS)_2$_E_LL-37::Nos-t**

Barley B1 hordein gene promoter: 13 bp – 562 bp

Kozak consensus sequence: 581 bp – 589 bp

ZmCKX1signal peptide: 590 bp – 640 bp

$(GGGGS)_2$: 641 bp – 670 bp, 689 bp – 718 bp

6xHis tag: 671 bp – 688 bp

Enterokinase restriction enzyme cut site: 719 bp – 733 bp

LL-37: 734 bp – 844 bp

Stop codon: 845 bp – 847 bp

Nos terminator: 872 bp – 1155 bp

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         10        20        30        40        50        60        70        80        90       100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTTAAAGTCGACGTGCAGGTGTATGAGTCATTGTTATGATCTATAGGTGTCAGTTTATCTTATCATCTGGGTGATCAATACAGGCCCAGGTTTTATAAAA

        110       120       130       140       150       160       170       180       190       200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACCAGTCGAGTCGAGAAGAACCGTCCACATGTAAAGCTTTAACAACCCACACATTGATTGCAACTTAGTCCTACACAAGTTTTCCATTCTTGTTTCAGGC

        210       220       230       240       250       260       270       280       290       300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TAACAACCTATACAAGGTTCCAAAATCATGCAAAAGTGATGCTAGGTTGATAATGTGTGACATGTAAAGTGAATAAGGTGAGTCATGCATACCAAACCTC

        310       320       330       340       350       360       370       380       390       400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GGGATTTCTATACTTTGTGTATGATCATATGCACAACTAAAAGGCAACTTTGATTATCAATTGAAAAGTACCGCTTGTAGCTTGTGCAACCTAACACAAT

        410       420       430       440       450       460       470       480       490       500
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTCCAAAAATCCATTTGCAAAAGCATCCAAACACAATTGTTAAAGCTGTTCAAACAAACAAAGAAGAGATGAAGCCTGGCTACTATAAATAGGCAGGTAG

        510       520       530       540       550       560       570       580       590       600
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TATAGAGATCTACACAAGCACAAGCATCAAAACCAAGAAACACTAGTTAACACCAATCCACTGTCGACGGTACCGGATCCGCCACCATGGCCGTTGTTTA

        610       620       630       640       650       660       670       680       690       700
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTACCTCCTCCTCGCCGGCCTCATTGCCTGCTCCCACGCTGGCGGCGGCGGCTCCGGCGGCGGGGGCAGCCACCATCACCATCATCATGGCGGGGGCGGC

        710       720       730       740       750       760       770       780       790       800
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AGCGGGGGGGGCGGCTCAGATGACGACGACAAGCTCCTTGGCGACTTCTTCCGCAAGTCCAAGGAGAAGATCGGCAAGGAGTTCAAGAGGATCGTCCAGA

        810       820       830       840       850       860       870       880       890       900
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GGATCAAGGACTTCCTGAGGAACCTCGTGCCGAGGACCGAGTCTTGACTCGAGCCTTGGTCTAGAGAGCTCAGTCAAGCAGGATCGTTCAAACATTTGGC

        910       920       930       940       950       960       970       980       990
1000
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATG

        1010      1020      1030      1040      1050      1060      1070      1080      1090
1100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGATAAA

        1110      1120      1130      1140      1150      1160      1170      1180
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
TTATCGCGCGCGGTGTCATCTATGTTACTAGATCGACCGGCATGCAAGCTGATATGAGCTCGCATGCGCGGCCGCGATATC
```

D) **<u>UBIp::ZmCKX1sp_(GGGGS)$_2$_6xHis_(GGGGS)$_2$_E_LL-37::Nos-t</u>**

Maize ubiquitin promoter: 1 bp – 1987 bp

Kozak consensus sequence: 2056 bp – 2064 bp

ZmCKX1signal peptide: 2065 bp – 2115 bp

(GGGGS)$_2$: 2116 bp – 2145 bp, 2164 bp – 2193 bp

6xHis tag: 2146 bp – 2163 bp

Enterokinase restriction enzyme cut site: 2194 bp – 2208 bp

LL-37: 2209 bp – 2319 bp

Stop codon: 2320 bp – 2322 bp

Nos terminator: 2416 bp – 2699 bp

```
         10        20        30        40        50        60        70        80        90       100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTGCAGCGTGACCCGGTCGTGCCCCTCTCTAGAGATAATGAGCATTGCATGTCTAAGTTATAAAAAATTACCACATATTTTTTTTGTCACACTTGTTTGA

        110       120       130       140       150       160       170       180       190       200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AGTGCAGTTTATCTATCTTTATACATATATTTAAACTTTACTCTACGAATAATATAATCTATAGTACTACAATAATATCAGTGTTTTAGAGAATCATATA

        210       220       230       240       250       260       270       280       290       300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AATGAACAGTTAGACATGGTCTAAAGGACAATTGAGTATTTTGACAACAGGACTCTACAGTTTTATCTTTTTAGTGTGCATGTGTTCTCCTTTTTTTTTG

        310       320       330       340       350       360       370       380       390       400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CAAATAGCTTCACCTATATAATACTTCATCCATTTTATTAGTACATCCATTTAGGGTTTAGGGTTAATGGTTTTTATAGACTAATTTTTTTAGTACATCT

        410       420       430       440       450       460       470       480       490       500
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATTTTATTCTATTTTAGCCTCTAAATTAAGAAAACTAAAACTCTATTTTAGTTTTTTTATTTAATAATTTAGATATAAAATAGAATAAAATAAAGTGACT

        510       520       530       540       550       560       570       580       590       600
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AAAAATTAAACAAATACCCTTTAAGAAATTAAAAAAACTAAGGAAACATTTTTCTTGTTTCGAGTAGATAATGCCAGCCTGTTAAACGCCGTCGACGAGT

        610       620       630       640       650       660       670       680       690       700
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTAACGGACACCAACCAGCGAACCAGCAGCGTCGCGTCGGGCCAAGCGAAGCAGACGGCACGGCATCTCTGTCGCTGCCTCTGGACCCCTCTCGAGAGTT

        710       720       730       740       750       760       770       780       790       800
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCGCTCCACCGTTGGACTTGCTCCGCTGTCGGCATCCAGAAATTGCGTGTCGGACGGCAGACGTGAGCCGGCACGGCAGGCGGCCTCCTCCTCCTCTCAC

        810       820       830       840       850       860       870       880       890       900
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GGCACCGGCAGCTACGGGGGATTCCTTTCCCACCGCTCCTTCGCTTTCCCTTCCTCGCCCGCCGTAATAAATAGACACCCCCTCCACACCCTCTTTCCCC

        910       920       930       940       950       960       970       980       990
1000
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AACCTCGTGTTGTTCGGAGCGCACACACACACAACCAGATCTCCCCCAAATCCACCCGTCGGCACCTCCGCTTCAAGGTACGCCGCTCGTCCTCCCCCCC

       1010      1020      1030      1040      1050      1060      1070      1080      1090
1100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCCCCCTCTCTACCTTCTCTAGATCGGCGTTCCGGTCCATGGTTAGGGCCCGGTAGTTCTACTTCTGTTCATGTTTGTGTTAGATCCGTGTTTGTGTTAG

       1110      1120      1130      1140      1150      1160      1170      1180      1190
1200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATCCGTGCTGCTAGCGTTCGTACACGGATGCGACCTGTACGTCAGACACGTTCTGATTGCTAACTTGCCAGTGTTTCTCTTTGGGGAATCCTGGGATGGC

       1210      1220      1230      1240      1250      1260      1270      1280      1290
1300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCTAGCCGTTCCGCAGACGGGATCGATTTCATGATTTTTTTTTGTTTCGTTGCATAGGGTTTGGTTTGCCCTTTTCCTTTATTTCAATATATGCCGTGCA

       1310      1320      1330      1340      1350      1360      1370      1380      1390
1400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
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CTTGTTTGTCGGGTCATCTTTTCATGCTTTTTTTTGTCTTGGTTGTGATGATGTGGTCTGGTTGGGCGGTCGTTCTAGATCGGAGTAGAATTAATTCTGT

       1410      1420      1430      1440      1450      1460      1470      1480      1490
1500
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTCAAACTACCTGGTGGATTTATTAATTTTGGATCTGTATGTGTGTGCCATACATATTCATAGTTACGAATTGAAGATGATGGATGGAAATATCGATCTA

       1510      1520      1530      1540      1550      1560      1570      1580      1590
1600
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GGATAGGTATACATGTTGATGCGGGTTTTACTGATGCATATACAGAGATGCTTTTTGTTCGCTTGGTTGTGATGATGTGGTGTGGTTGGGCGGTCGTTCA

       1610      1620      1630      1640      1650      1660      1670      1680      1690
1700
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTCGTTCTAGATCGGAGTAGAATACTGTTTCAAACTACCTGGTGTATTTATTAATTTTGGAACTGTATGTGTGTGTCATACATCTTCATAGTTACGAGTT

       1710      1720      1730      1740      1750      1760      1770      1780      1790
1800
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TAAGATGGATGGAAATATCGATCTAGGATAGGTATACATGTTGATGTGGGTTTTACTGATGCATATACATGATGGCATATGCAGCATCTATTCATATGCT

       1810      1820      1830      1840      1850      1860      1870      1880      1890
1900
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTAACCTTGAGTACCTATCTATTATAATAAACAAGTATGTTTTATAATTATTTTGATCTTGATATACTTGGATGATGGCATATGCAGCAGCTATATGTGG

       1910      1920      1930      1940      1950      1960      1970      1980      1990
2000
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATTTTTTTAGCCCTGCCTTCATACGCTATTTATTTGCTTGGTACTGTTTCTTTTGTCGATGCTCACCCTGTTGTTTGGTGTTACTTCGCCCATCACAAGT

       2010      2020      2030      2040      2050      2060      2070      2080      2090
2100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTGTACAAAAAAGCAGGCTGGCGCCGGAACCAATTCAGTCGACCGTACGGGATCCGCCACCATGGCCGTTGTTTACTACCTCCTCCTCGCCGGCCTCATT

       2110      2120      2130      2140      2150      2160      2170      2180      2190
2200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GCCTGCTCCCACGCTGGCGGCGGCGGCTCCGGCGGCGGGGGGCAGCCACCATCACCATCATCATGGCGGGGGGCGGCAGCGGGGGGGGCGGCTCAGATGACG

       2210      2220      2230      2240      2250      2260      2270      2280      2290
2300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACGACAAGCTCCTTGGCGACTTCTTCCGCAAGTCCAAGGAGAAGATCGGCAAGGAGTTCAAGAGGATCGTCCAGAGGATCAAGGACTTCCTGAGGAACCT

       2310      2320      2330      2340      2350      2360      2370      2380      2390
2400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CGTGCCGAGGACCGAGTCTTGACTCGAGGAATTCGCGGCCGCACTCGAGATATCTAGACCCAGCTTTCTTGTACAAAGTGGTGATGGGGGATCCACTAGT

       2410      2420      2430      2440      2450      2460      2470      2480      2490
2500
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCTAGAATTCGATTGAGTCAAGCAGGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAA

       2510      2520      2530      2540      2550      2560      2570      2580      2590
2600
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTT

       2610      2620      2630      2640      2650      2660      2670      2680      2690
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....
AATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCGACCGGCATGCAAGCTGATAT
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E) **B-HORp::ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL::Nos-t**

Barley B1 hordein gene promoter: 13 bp – 562 bp

Kozak consensus sequence: 581 bp – 592 bp

ZmCKX1signal peptide: 593 bp – 643 bp

6xHis tag: 644 bp – 661 bp

Maltose binding protein: 662 bp – 1807 bp

Enterokinase restriction enzyme cut site: 1808 bp – 1822 bp

LL-37: 1823 bp – 1933 bp

KDEL signal peptide: 1934 bp – 1945 bp

Stop codon: 1946 bp – 1948 bp

Nos terminator: 1973 bp – 2256 bp

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         10        20        30        40        50        60        70        80        90       100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTTAAAGTCGACGTGCAGGTGTATGAGTCATTGTTATGATCTATAGGTGTCAGTTTATCTTATCATCTGGGTGATCAATACAGGCCCAGGTTTTATAAAA

        110       120       130       140       150       160       170       180       190       200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACCAGTCGAGTCGAGAAGAACCGTCCACATGTAAAGCTTTAACAACCCACACATTGATTGCAACTTAGTCCTACACAAGTTTTCCATTCTTGTTTCAGGC

        210       220       230       240       250       260       270       280       290       300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TAACAACCTATACAAGGTTCCAAAATCATGCAAAAGTGATGCTAGGTTGATAATGTGTGACATGTAAAGTGAATAAGGTGAGTCATGCATACCAAACCTC

        310       320       330       340       350       360       370       380       390       400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GGGATTTCTATACTTTGTGTATGATCATATGCACAACTAAAAGGCAACTTTGATTATCAATTGAAAAGTACCGCTTGTAGCTTGTGCAACCTAACACAAT

        410       420       430       440       450       460       470       480       490       500
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTCCAAAAATCCATTTGCAAAAGCATCCAAACACAATTGTTAAAGCTGTTCAAACAAACAAAGAAGAGATGAAGCCTGGCTACTATAAATAGGCAGGTAG

        510       520       530       540       550       560       570       580       590       600
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TATAGAGATCTACACAAGCACAAGCATCAAAACCAAGAAACACTAGTTAACACCAATCCACTGTCGACGGTACCGGATCCGCGGCAGCGATGGCCGTGGT

        610       620       630       640       650       660       670       680       690       700
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTACTACCTCCTCCTCGCCGGCCTGATCGCCTGCTCCCACGCGCACCACCACCATCACCATAAGATCGAGGAGGGCAAGCTCGTCATCTGGATCAACGGC

        710       720       730       740       750       760       770       780       790       800
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GACAAGGGCTACAACGGCCTCGCCGAGGTGGGCAAGAAGTTCGAGAAGGACACCGGCATCAAGGTGACCGTCGAGCACCCAGATAAGCTGGAGGAGAAGT

        810       820       830       840       850       860       870       880       890       900
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCCCGCAGGTCGCCGCCACCGGCGACGGCCCGGATATCATCTTCTGGGCCCACGACAGGTTCGGCGGCTACGCCCAGTCCGGCCTCCTCGCCGAGATCAC

        910       920       930       940       950       960       970       980       990
1000
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCCGGACAAGGCCTTCCAGGATAAGCTCTACCCGTTCACCTGGGACGCCGTGAGGTACAACGGCAAGCTCATTGCCTACCCGATTGCCGTCGAGGCCCTC

       1010      1020      1030      1040      1050      1060      1070      1080      1090
1100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCCCTGATCTACAACAAGGACCTCCTCCCGAACCCGCCCAAGACCTGGGAGGAGATCCCGGCCCTCGACAAGGAGCTGAAGGCCAAGGGCAAGTCCGCCC

       1110      1120      1130      1140      1150      1160      1170      1180      1190
1200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCATGTTCAACCTCCAGGAGCCGTACTTCACCTGGCCACTCATTGCGGCCGACGGGGGGTACGCCTTCAAGTACGAGAACGGCAAGTACGACATCAAGGA

       1210      1220      1230      1240      1250      1260      1270      1280      1290
1300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CGTGGGCGTGGACAACGCTGGCGCCAAGGCGGGCCTCACCTTCCTCGTGGATCTCATCAAGAACAAGCACATGAACGCCGACACCGACTACTCGATCGCC
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        1310      1320      1330      1340      1350      1360      1370      1380      1390
1400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GAGGCCGCCTTCAACAAGGGCGAGACCGCCATGACCATTAACGGCCCGTGGGCCTGGTCCAACATCGACACCTCTAAGGTGAACTACGGCGTGACCGTGC

        1410      1420      1430      1440      1450      1460      1470      1480      1490
1500
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCCCGACCTTCAAGGGCCAGCCGTCCAAGCCGTTCGTGGGCGTGCTCTCCGCCGGCATCAACGCCGCCTCCCCGAACAAGGAGCTGGCCAAGGAGTTCCT

        1510      1520      1530      1540      1550      1560      1570      1580      1590
1600
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GGAGAACTACCTCCTCACCGACGAGGGCCTGGAGGCCGTGAACAAGGACAAGCCGCTCGGCGCCGTGGCCCTCAAGTCCTACGAGGAGGAGCTGGCGAAG

        1610      1620      1630      1640      1650      1660      1670      1680      1690
1700
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GACCCGAGGATCGCCGCGACCATGGAGAACGCCCAGAAGGGCGAGATCATGCCGAACATCCCGCAGATGTCCGCCTTCTGGTACGCCGTCAGGACCGCCG

        1710      1720      1730      1740      1750      1760      1770      1780      1790
1800
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TGATTAACGCCGCCAGCGGCAGGCAGACCGTGGACGAGGCCCTGAAGGACGCCCAGACCAACTCTTCCTCGAACAATAACAATAACAACAATAATAACAA

        1810      1820      1830      1840      1850      1860      1870      1880      1890
1900
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCTCGGCGACGACGACGACAAGCTCCTCGGCGACTTCTTCCGCAAGTCCAAGGAGAAGATCGGCAAGGAGTTCAAGAGGATCGTCCAGAGGATCAAGGAC

        1910      1920      1930      1940      1950      1960      1970      1980      1990
2000
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTCCTCCGCAACCTCGTGCCAAGGACCGAGTCCAAGGATGAGCTGTGACTCGAGCCTTGGTCTAGAGAGCTCAGTCAAGCAGGATCGTTCAAACATTTGG

        2010      2020      2030      2040      2050      2060      2070      2080      2090
2100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAAT

        2110      2120      2130      2140      2150      2160      2170      2180      2190
2200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGATAA

        2210      2220      2230      2240      2250      2260      2270      2280
....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
ATTATCGCGCGCGGTGTCATCTATGTTACTAGATCGACCGGCATGCAAGCTGATATGAGCTCGCATGCGCGGCCGCGATATC
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F) **<u>UBIp::ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL::Nos-t</u>**

Maize ubiquitin promoter: 1 bp – 1987 bp

Kozak consensus sequence: 2056 bp – 2067 bp

ZmCKX1signal peptide: 2068 bp – 2118 bp

6xHis tag: 2119 bp – 2136 bp

Maltose binding protein: 2137 bp – 3282 bp

Enterokinase restriction enzyme cut site: 3283 bp – 3297 bp

LL-37: 3298 bp – 3408 bp

KDEL signal peptide: 3409 bp – 3420 bp

Stop codon: 3421 bp – 3423 bp

Nos terminator: 3517 bp – 3800 bp

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         10        20        30        40        50        60        70        80        90       100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTGCAGCGTGACCCGGTCGTGCCCCTCTCTAGAGATAATGAGCATTGCATGTCTAAGTTATAAAAAATTACCACATATTTTTTTTGTCACACTTGTTTGA

        110       120       130       140       150       160       170       180       190       200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AGTGCAGTTTATCTATCTTTATACATATATTTAAACTTTACTCTACGAATAATATAATCTATAGTACTACAATAATATCAGTGTTTTAGAGAATCATATA

        210       220       230       240       250       260       270       280       290       300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AATGAACAGTTAGACATGGTCTAAAGGACAATTGAGTATTTTGACAACAGGACTCTACAGTTTTATCTTTTTAGTGTGCATGTGTTCTCCTTTTTTTTTG

        310       320       330       340       350       360       370       380       390       400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CAAATAGCTTCACCTATATAATACTTCATCCATTTTATTAGTACATCCATTTAGGGTTTAGGGTTAATGGTTTTTATAGACTAATTTTTTTTAGTACATCT

        410       420       430       440       450       460       470       480       490       500
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATTTTATTCTATTTTAGCCTCTAAATTAAGAAAACTAAAACTCTATTTTAGTTTTTTTATTTAATAATTTAGATATAAAATAGAATAAAATAAAGTGACT

        510       520       530       540       550       560       570       580       590       600
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AAAAATTAAACAAATACCCTTTAAGAAATTAAAAAAACTAAGGAAACATTTTTCTTGTTTCGAGTAGATAATGCCAGCCTGTTAAACGCCGTCGACGAGT

        610       620       630       640       650       660       670       680       690       700
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTAACGGACACCAACCAGCGAACCAGCAGCGTCGCGTCGGGCCAAGCGAAGCAGACGGCACGGCATCTCTGTCGCTGCCTCTGGACCCCTCTCGAGAGTT

        710       720       730       740       750       760       770       780       790       800
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCGCTCCACCGTTGGACTTGCTCCGCTGTCGGCATCCAGAAATTGCGTGTCGGACGGCAGACGTGAGCCGGCACGGCAGGCGGCCTCCTCCTCCTCTCAC

        810       820       830       840       850       860       870       880       890       900
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GGCACCGGCAGCTACGGGGGGATTCCTTTCCCACCGCTCCTTCGCTTTCCCTTCCTCGCCCGCCGTAATAAATAGACACCCCCTCCACACCCTCTTTCCCC

        910       920       930       940       950       960       970       980       990
1000
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AACCTCGTGTTGTTCGGAGCGCACACACACACAACCAGATCTCCCCCAAATCCACCCGTCGGCACCTCCGCTTCAAGGTACGCCGCTCGTCCTCCCCCCC

       1010      1020      1030      1040      1050      1060      1070      1080      1090
1100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCCCCCTCTCTACCTTCTCTAGATCGGCGTTCCGGTCCATGGTTAGGGCCCGGTAGTTCTACTTCTGTTCATGTTTGTGTTAGATCCGTGTTTGTGTTAG

       1110      1120      1130      1140      1150      1160      1170      1180      1190
1200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATCCGTGCTGCTAGCGTTCGTACACGGATGCGACCTGTACGTCAGACACGTTCTGATTGCTAACTTGCCAGTGTTTCTCTTTGGGGAATCCTGGGATGGC

       1210      1220      1230      1240      1250      1260      1270      1280      1290
1300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCTAGCCGTTCCGCAGACGGGATCGATTTCATGATTTTTTTTTGTTTCGTTGCATAGGGTTTGGTTTGCCCTTTTCCTTTATTTCAATATATGCCGTGCA
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        1310      1320      1330      1340      1350      1360      1370      1380      1390
1400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTTGTTTGTCGGGTCATCTTTTCATGCTTTTTTTTGTCTTGGTTGTGATGATGTGGTCTGGTTGGGCGGTCGTTCTAGATCGGAGTAGAATTAATTCTGT

        1410      1420      1430      1440      1450      1460      1470      1480      1490
1500
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTCAAACTACCTGGTGGATTTATTAATTTTGGATCTGTATGTGTGTGCCATACATATTCATAGTTACGAATTGAAGATGATGGATGGAAATATCGATCTA

        1510      1520      1530      1540      1550      1560      1570      1580      1590
1600
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GGATAGGTATACATGTTGATGCGGGTTTTACTGATGCATATACAGAGATGCTTTTTGTTCGCTTGGTTGTGATGATGTGGTGTGGTTGGGCGGTCGTTCA

        1610      1620      1630      1640      1650      1660      1670      1680      1690
1700
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTCGTTCTAGATCGGAGTAGAATACTGTTTCAAACTACCTGGTGTATTTATTAATTTTGGAACTGTATGTGTGTGTCATACATCTTCATAGTTACGAGTT

        1710      1720      1730      1740      1750      1760      1770      1780      1790
1800
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TAAGATGGATGGAAATATCGATCTAGGATAGGTATACATGTTGATGTGGGTTTTACTGATGCATATACATGATGGCATATGCAGCATCTATTCATATGCT

        1810      1820      1830      1840      1850      1860      1870      1880      1890
1900
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTAACCTTGAGTACCTATCTATTATAATAAACAAGTATGTTTTATAATTATTTTGATCTTGATATACTTGGATGATGGCATATGCAGCAGCTATATGTGG

        1910      1920      1930      1940      1950      1960      1970      1980      1990
2000
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATTTTTTTAGCCCTGCCTTCATACGCTATTTATTTGCTTGGTACTGTTTCTTTTGTCGATGCTCACCCTGTTGTTTGGTGTTACTTCGCCCATCACAAGT

        2010      2020      2030      2040      2050      2060      2070      2080      2090
2100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTGTACAAAAAAGCAGGCTGGCGCCGGAACCAATTCAGTCGACCGTACGGGATCCGCGGCAGCGATGGCCGTGGTGTACTACCTCCTCCTCGCCGGCCTG

        2110      2120      2130      2140      2150      2160      2170      2180      2190
2200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATCGCCTGCTCCCACGCGCACCACCACCATCACCATAAGATCGAGGAGGGCAAGCTCGTCATCTGGATCAACGGCGACAAGGGCTACAACGGCCTCGCCG

        2210      2220      2230      2240      2250      2260      2270      2280      2290
2300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AGGTGGGCAAGAAGTTCGAGAAGGACACCGGCATCAAGGTGACCGTCGAGCACCCAGATAAGCTGGAGGAGAAGTTCCCGCAGGTCGCCGCCACCGGCGA

        2310      2320      2330      2340      2350      2360      2370      2380      2390
2400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CGGCCCGGATATCATCTTCTGGGCCCACGACAGGTTCGGCGGCTACGCCCAGTCCGGCCTCCTCGCCGAGATCACCCCGGACAAGGCCTTCCAGGATAAG

        2410      2420      2430      2440      2450      2460      2470      2480      2490
2500
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTCTACCCGTTCACCTGGGACGCCGTGAGGTACAACGGCAAGCTCATTGCCTACCCGATTGCCGTCGAGGCCCTCTCCCTGATCTACAACAAGGACCTCC

        2510      2520      2530      2540      2550      2560      2570      2580      2590
2600
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCCCGAACCCGCCCAAGACCTGGGAGGAGATCCCGGCCCTCGACAAGGAGCTGAAGGCCAAGGGCAAGTCCGCCCTCATGTTCAACCTCCAGGAGCCGTA

        2610      2620      2630      2640      2650      2660      2670      2680      2690
2700
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTTCACCTGGCCACTCATTGCGGCCGACGGGGGGTACGCCTTCAAGTACGAGAACGGCAAGTACGACATCAAGGACGTGGGCGTGGACAACGCTGGCGCC

        2710      2720      2730      2740      2750      2760      2770      2780      2790
2800
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AAGGCGGGCCTCACCTTCCTCGTGGATCTCATCAAGAACAAGCACATGAACGCCGACACCGACTACTCGATCGCCGAGGCCGCCTTCAACAAGGGCGAGA

        2810      2820      2830      2840      2850      2860      2870      2880      2890
2900
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCGCCATGACCATTAACGGCCCGTGGGCCTGGTCCAACATCGACACCTCTAAGGTGAACTACGGCGTGACCGTGCTCCCGACCTTCAAGGGCCAGCCGTC

        2910      2920      2930      2940      2950      2960      2970      2980      2990
3000
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CAAGCCGTTCGTGGGCGTGCTCTCCGCCGGCATCAACGCCGCCTCCCCGAACAAGGAGCTGGCCAAGGAGTTCCTGGAGAACTACCTCCTCACCGACGAG

        3010      3020      3030      3040      3050      3060      3070      3080      3090
3100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
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GGCCTGGAGGCCGTGAACAAGGACAAGCCGCTCGGCGCCGTGGCCCTCAAGTCCTACGAGGAGGAGCTGGCGAAGGACCCGAGGATCGCCGCGACCATGG

        3110      3120      3130      3140      3150      3160      3170      3180      3190
3200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AGAACGCCCAGAAGGGCGAGATCATGCCGAACATCCCGCAGATGTCCGCCTTCTGGTACGCCGTCAGGACCGCCGTGATTAACGCCGCCAGCGGCAGGCA

        3210      3220      3230      3240      3250      3260      3270      3280      3290
3300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GACCGTGGACGAGGCCCTGAAGGACGCCCAGACCAACTCTTCCTCGAACAATAACAATAACAACAATAATAACAACCTCGGCGACGACGACGACAAGCTC

        3310      3320      3330      3340      3350      3360      3370      3380      3390
3400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTCGGCGACTTCTTCCGCAAGTCCAAGGAGAAGATCGGCAAGGAGTTCAAGAGGATCGTCCAGAGGATCAAGGACTTCCTCCGCAACCTCGTGCCAAGGA

        3410      3420      3430      3440      3450      3460      3470      3480      3490
3500
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCGAGTCCAAGGATGAGCTGTGACTCGAGGAATTCGCGGCCGCACTCGAGATATCTAGACCCAGCTTTCTTGTACAAAGTGGTGATGGGGGATCCACTAG

        3510      3520      3530      3540      3550      3560      3570      3580      3590
3600
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTCTAGAATTCGATTGAGTCAAGCAGGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATA

        3610      3620      3630      3640      3650      3660      3670      3680      3690
3700
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATT

        3710      3720      3730      3740      3750      3760      3770      3780      3790
3800
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TAATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCGACCGGCATGCAAGCTGATAT
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G) **B-HORp::OsCht1sp_6xHis_E_LL-37::Nos-t**

Barley B1 hordein gene promoter: 13 bp – 562 bp

Kozak consensus sequence: 581 bp – 589 bp

OsCht1signal peptide: 590 bp – 646 bp

6xHis tag: 647 bp – 664 bp

Enterokinase restriction enzyme cut site: 665 bp – 679 bp

LL-37: 680 bp – 790 bp

Stop codon: 791 – 793

Nos terminator: 818 bp – 1101 bp

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         10        20        30        40        50        60        70        80        90       100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTTAAAGTCGACGTGCAGGTGTATGAGTCATTGTTATGATCTATAGGTGTCAGTTTATCTTATCATCTGGGTGATCAATACAGGCCCAGGTTTTATAAAA

        110       120       130       140       150       160       170       180       190       200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACCAGTCGAGTCGAGAAGAACCGTCCACATGTAAAGCTTTAACAACCCACACATTGATTGCAACTTAGTCCTACACAAGTTTTCCATTCTTGTTTCAGGC

        210       220       230       240       250       260       270       280       290       300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TAACAACCTATACAAGGTTCCAAAATCATGCAAAAGTGATGCTAGGTTGATAATGTGTGACATGTAAAGTGAATAAGGTGAGTCATGCATACCAAACCTC

        310       320       330       340       350       360       370       380       390       400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GGGATTTCTATACTTTGTGTATGATCATATGCACAACTAAAAGGCAACTTTGATTATCAATTGAAAAGTACCGCTTGTAGCTTGTGCAACCTAACACAAT

        410       420       430       440       450       460       470       480       490       500
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTCCAAAAATCCATTTGCAAAAGCATCCAAACACAATTGTTAAAGCTGTTCAAACAAACAAAGAAGAGATGAAGCCTGGCTACTATAAATAGGCAGGTAG

        510       520       530       540       550       560       570       580       590       600
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TATAGAGATCTACACAAGCACAAGCATCAAAACCAAGAAACACTAGTTAACACCAATCCACTGTCGACGGTACCGGATCCGCCACCATGAGGGCTCTCGC

        610       620       630       640       650       660       670       680       690       700
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CGTGGTGGTGGTCGCCACCGCCTTCGCTGTGGTGGCCGTGAGGGGCCACCATCACCATCATCATGACGACGACGATAAGCTCCTCGGCGACTTCTTCCGC

        710       720       730       740       750       760       770       780       790       800
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AAGTCCAAGGAGAAGATCGGCAAGGAGTTCAAGAGGATCGTCCAGAGGATCAAGGACTTCCTGAGGAACCTCGTGCCGAGGACCGAGTCTTGACTCGAGC

        810       820       830       840       850       860       870       880       890       900
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTTGGTCTAGAGAGCTCAGTCAAGCAGGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATAT

        910       920       930       940       950       960       970       980       990
1000
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACAT

       1010      1020      1030      1040      1050      1060      1070      1080      1090
1100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTAATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCGACCGGCATGCAAGCTGATA

       1110      1120
....|....|....|....|...
TGAGCTCGCATGCGCGGCCGCGATATC
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H) **<u>TIp::PEX_KDEL::Nos-t</u>**

*Att*L1: 1 bp – 100 bp

Barley trypsin inhibitor promoter: 101 bp – 2493 bp

Kozak consensus sequence: 2506 bp – 2514 bp

Pexiganan: 2515 bp – 2580 bp

KDEL signal peptide: 2581 bp – 2592 bp

Stop codon: 2593 bp – 2595 bp

Nos terminator: 2608 bp – 2857 bp

*Att*L2: 2858 bp – 2957 bp

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         10        20        30        40        50        60        70        80        90       100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTGATAAGCAATGCTTTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCT

        110       120       130       140       150       160       170       180       190       200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTCGACGCATGTGTCATCTCATTCTGAAACGTGTGGTGCTGAGACGGTTGGAAATATGCCCTAGAGGTAATGATAAATAGTTATTATTATATTTCTTGTT

        210       220       230       240       250       260       270       280       290       300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCAAGATAATCATTTATTATTCATGCTATAATTATATTGAATGAAAACATAGATACATGTGTGAATACATTGACGAAACAATGTCCTTAGCAAGCCTCTA

        310       320       330       340       350       360       370       380       390       400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTTGGCTAGCCAGTTGATCAAGGATAGTCAAGGTTTTCTGACTATGTGCAAGTGTTGTCACTTGATAACTGGATCACATCATTAGGAGAATCATGTGATG

        410       420       430       440       450       460       470       480       490       500
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GACTAGACCCAAACTATGAATGTAGCATATTGATCGTGTCATTTTGTTGCTATTGTTTTCTGCGTGTCAAGTATTTATTCCTATGACCATGAGATCATAT

        510       520       530       540       550       560       570       580       590       600
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AACTCACTGGTACCGGAGGAATACCTTGTGTGTATCAAACGTCGCAACGTAACTGAGTGACTATAAAGGTGCTCTACAGGTATCTCTGAAGGTGTCCGTT

        610       620       630       640       650       660       670       680       690       700
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GAGTTAGTATGGATCAAGACTAGGATTTGCCACTCCGTGTGACGTAGAGGTATCTCGGCCCACTCGGTAATACAACATCACACACAAGCCTTGCAAGCAA

        710       720       730       740       750       760       770       780       790       800
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TGTGACTAAGTTTAAGTCACGGGATCTTGTATTACCGAACGAGAAAAGAGACATGGATGCTAGTAGATTTCTTTTTGTAGAGCACCTCACAACTAGACGG

        810       820       830       840       850       860       870       880       890       900
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GCCCTGAAACACCTATACTTATATTTAGTATACAAAATGTTTGTCCTGAAACAATACGTGAGTTGCAGAAAACGAAGCGACTTCGATAGACGCAGGAACA

        910       920       930       940       950       960       970       980       990
1000
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CACGATGGAACGACCACGGAGCCGAAGGACGGCCGCGCGATCGGTCGGTACTAGATAATATTTTCCCAAAAGATAATGCATGGATGCTAGTAGACTTATT

       1010      1020      1030      1040      1050      1060      1070      1080      1090
1100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTCAGAAGTACTATACATACGATACGATACGACATATCATACCAAAATCGTATGCCAAATTATCGTATATAGAGTAATTCCGTTTCTTTTTTGTCCCGGT

       1110      1120      1130      1140      1150      1160      1170      1180      1190
1200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GGATCTGAAATAACCACTTTCTGGAAAATCTTGGAAATTTAGGTTTTTTCTGCCCGAATGAATTTAAAGGAACTTTGAATTTGATTTTTTTGCTGTGTGA

       1210      1220      1230      1240      1250      1260      1270      1280      1290
1300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AAATATATATAAAGACGCTAACCTACTGACTAACGTAACTCCCTCATGCTCGACTGATCGGTTGGGTACACTCTTTCGCCCGCCAGGTCATGAGATTGGA

       1310      1320      1330      1340      1350      1360      1370      1380      1390
1400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCCTCTCGATCGGTTGCAGACATGATCTCGACTCATAAACCAAACGAGAGTTTACTTGAAGTGCTAGATTACACTACAAATAAACATGGAAGAGAATGAC
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        1410      1420      1430      1440      1450      1460      1470      1480      1490
1500
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATGCAGATGAACTAGGGATAATTCTGTTATCTCTTTTCGTAGGGTTCTTCTTGAACCTCGACTGGCAGCATGGGATGAAGTACTCACTCGTTTGGAGGCT

        1510      1520      1530      1540      1550      1560      1570      1580      1590
1600
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CGTTCAGCTGTCACATGGATGCTAGTAGATTTCTTTTCGTAGAGCCCCTCACAACCAGACGGGCCCCGAAACACCTATACTTATATCCAGTATAAAAAAT

        1610      1620      1630      1640      1650      1660      1670      1680      1690
1700
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTCTGTCCTGAAACAATACGTGAGTTGCAGGAAAACGAAAGCGGCTTCGATAGACGCAGGAACACACGAACGAACGGCCGCGCGATTGGTCGGTACTAGA

        1710      1720      1730      1740      1750      1760      1770      1780      1790
1800
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TAATATTTTCCCAAAAGATAATGCATGGATGCTACTAGATTTTTTTCAGAAGTACTCTGCATACGATACGATATGACATATCATACAAAATCGTATGCCA

        1810      1820      1830      1840      1850      1860      1870      1880      1890
1900
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AATTATCGTATTTAGAGTTAATTATGTTTCTTTTTTGTTCGAGGGATCTGAAATAACCGGTTTCTGGAAAATCTTGTAAATTCAAGGTTTTTCCGCCCGA

        1910      1920      1930      1940      1950      1960      1970      1980      1990
2000
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATGAATTTAAACAAACTTTGAATTTGATTTTTTTGCAGTGTGAAAATATATATAAAGATGCTAACCTACTGACTAACGTAACTCCCTCATGCTCGACTGA

        2010      2020      2030      2040      2050      2060      2070      2080      2090
2100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCGGTTGCGTAGACCCTGTCGCCCGCCAGGTCATGAGATTGGATCCTCTCGGCTGCAGACATGATCTCCACTCATAAAGCAAACGAGAGTTTACTTGAAG

        2110      2120      2130      2140      2150      2160      2170      2180      2190
2200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TGTTAGATTACACATTTACACTACGAATAAACATGGAAGAGAATGGCATGCAGATGAACCAGGGATGTAGCGTAGTATGATAAGATAACTCATGCCAAGC

        2210      2220      2230      2240      2250      2260      2270      2280      2290
2300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACACTGAGAAAGGTAGGAACCGAGGAAATATGGTTATTCTTCTACTCAAACCCCAACTTTTGCATTCCCCTCCGAATATTGTTAGCACAAAGCTCCAAAT

        2310      2320      2330      2340      2350      2360      2370      2380      2390
2400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCTGTCAGCAAAAAAGGTTATCAAAGCTACCCTTTAATCAAAATTTCCATGCAGCGGCACTCCAACAACTAACAGAAAGTCAGAAAGCACTTCGGTACTT

        2410      2420      2430      2440      2450      2460      2470      2480      2490
2500
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACGATGCCAGCTGCTTGTCACTTCACATTTCACTATATATATACATGTGATACGCCTCGCTTGCTCCAACAACATCCCTTCAATTTGATAACACGTACGG

        2510      2520      2530      2540      2550      2560      2570      2580      2590
2600
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AATTCGCCACCATGGGCATCGGCAAGTTCCTCAAGAAGGCCAAGAAGTTCGGCAAGGCCTTCGTGAAGATCCTGAAGAAGAAGGACGAGCTTTGACTCGA

        2610      2620      2630      2640      2650      2660      2670      2680      2690
2700
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GCCTAGGTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACGTTAAGCAT

        2710      2720      2730      2740      2750      2760      2770      2780      2790
2800
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATAT

        2810      2820      2830      2840      2850      2860      2870      2880      2890
2900
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCGGGACCCAGCTTTCTTGTACAAAGTTGGCATTATAAGAAAGCATTG

        2910      2920      2930      2940      2950
....|....|....|....|....|....|....|....|....|..
CTTATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAAAATAAAATCATTATTTG
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I) **AsGLO1p::8x His_HBD2::Nos-t**

*Att*L1: 1 bp – 100 bp

Oat globulin promoter: 107 bp – 1066 bp

16bp untranslated leader sequence: 1067 bp – 1082 bp

Kozak consensus sequence: 1095 bp – 1103 bp

8xHis tag: 1104 bp – 1127 bp

Enterokinase restriction enzyme cut site: 1128 bp – 1142 bp

Human beta-defensin 2: 1143 bp – 1259 bp

Stop codon: 1260 bp – 1262 bp

Nos terminator: 1275 bp – 1524 bp

*Att*L2: 1525 bp – 1624 bp

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        10        20        30        40        50        60        70        80        90       100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTGATAAGCAATGCTTTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCT

        110       120       130       140       150       160       170       180       190       200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTCGACTGGAAAGTCATTTTGCCTCCTGAACTCCAGTGTTTCCTGTTTATTAAAAAAACTAAAAACTATACTTATAAGTTTGAAAAGATCATGAAACAAA

        210       220       230       240       250       260       270       280       290       300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATTGTAAAAATTGCTAATGATATATCCCACAAACGTGCAAAATCTCAATTCGAAGTGCTTTGTATTTCGAACTACACAAAAATGACAAAGTGTGACTTTT

        310       320       330       340       350       360       370       380       390       400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TATGTGATTCGAAATCACTATACTACAGATCTACAATTTTGTCATTTTTGTGAAGCTAAAAATACACATTATTTCGAATTGAGATTTTTCATGTTTGTGC

        410       420       430       440       450       460       470       480       490       500
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TATGAATCACAGGCTACATCCTGATTTATTTTTAGAATTTTTTGGAACCCAAAATATGTTCTAGATTATTTTTTAAAAAGTGGGATCACTTATGCCCATA

        510       520       530       540       550       560       570       580       590       600
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CACACGAAATCTCCACTCAATTCTTTTATACATTATCTTTCTATATCTACTAACGTGGATTATACATCATAGTAAGTTTCTTACTACATGTGCTTTCTTG

        610       620       630       640       650       660       670       680       690       700
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TGACAATGTGGACACGACTCTTCCACTTTTGGGCTTTATGTTGTATTGATATACTCATGACATGGAATTTTGTCCACACACGTAGATCCATCCATATATA

        710       720       730       740       750       760       770       780       790       800
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTGTTGTGCATAGAACGAAACACAAGCAAGCCATTAAAAAGGAGTCACAAGTGCCACAAACTGTTGTAGGAAGTACAACTAGTATGAGGCCTTTTATTTG

        810       820       830       840       850       860       870       880       890       900
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACGTCGGACAATGGCCAAGAGCTACATACAAAAGATGGTGCTAGATTTGTGAGTAAGCACCAGTTGTAGGCAGAAAACAACACATATCTTTTGAGGCAAA

        910       920       930       940       950       960       970       980       990
1000
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTTATATCTATTCACATTTAAAACCATGATCTGTTGAGTCACCATGAATATCTTTTTATCTATGTTAATAATTACATGTCATCATGTTTATCCTGGACTA

       1010      1020      1030      1040      1050      1060      1070      1080      1090
1100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTTTTTATGGCTATAAAATCAAACTTACAATTAGGAAACTAGCACCAATCCACCTTCTACAATCTCGGATCCGTCCTAAAGCCGTACGGAATTCGCCACC

       1110      1120      1130      1140      1150      1160      1170      1180      1190
1200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATGCACCACCATCATCATCACCATCACGACGACGATGACAAGGACCCGGTGACCTGCCTCAAGTCTGGCGCCATTTGCCACCCAGTGTTCTGCCCAAGGC

       1210      1220      1230      1240      1250      1260      1270      1280      1290
1300
```

```
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GCTACAAGCAGATCGGCACGTGCGGCCTCCCAGGCACCAAGTGCTGCAAGAAGCCAAACTGACTCGAGCCTAGGTCAAACATTTGGCAATAAAGTTTCTT

        1310      1320      1330      1340      1350      1360      1370      1380      1390
1400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTT

        1410      1420      1430      1440      1450      1460      1470      1480      1490
1500
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGG

        1510      1520      1530      1540      1550      1560      1570      1580      1590
1600
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TGTCATCTATGTTACTAGATCGGGACCCAGCTTTCTTGTACAAAGTTGGCATTATAAGAAAGCATTGCTTATCAATTTGTTGCAACGAACAGGTCACTAT

        1610      1620
....|....|....|....|....
CAGTCAAAATAAAATCATTATTTG
```

Figure S2. Nucleotide sequences of individual functional fragments and their positions in the designed expression vectors used in this study for recombinant production of indicated antimicrobial peptides.

PALACKÝ UNIVERSITY OLOMOUC

Faculty of Science

SUMMARY OF THE DOCTORAL THESIS

Generation and analysis of new transgenic barley lines expressing recombinant antimicrobial peptides.

P1416 Biochemistry

Edita Holásková

Olomouc 2019

The experiments included in this Ph.D. thesis have been performed in the laboratories of Department of Molecular Biology, Centre of the Region Haná for Biotechnological and Agricultural Research (Palacký University Olomouc) under the supervision of doc. Mgr. Petr Galuszka†, Ph.D. and prof. RNDr. Ivo Frébort, CSc., Ph.D. in the period 2012-2017.

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Table of content

Abstract

An alarming increase in the emergence of antibiotic resistant strains of bacteria presents a severe problem at the global scale that requires an urgent action to avoid the so called post-antibiotic era, in which bacteria may became completely resistant to treatment, thus common infections could once again kill. Antimicrobial peptides (AMPs) LL-37, beta-defensin 2, and pexiganan, represent promising control agents to treat drug-resistant infections, as they target a wide spectrum of pathogens, including those of medical importance. Despite their therapeutic potential, the use of AMPs in medicine is limited, mainly due to their high production costs. In general, the use of plants for their production seems to be beneficial in this respect. However, certain technical limitations still remain. These are basically connected to potential toxicity of AMPs to the host plant, low accumulation levels and eventually also to the issues connected to product isolation and its biological activity.

In an effort to address the above mentioned challenges, several fusion protein or peptide translocation, stabilization and purification sequences were selected and tested for their impact on accumulation level of LL-37 using transient expression in tobacco leaves. Results of this analysis provided valuable data that were taken into account when designing constructs for heterologous expression of LL-37 in barley.

Next, stable transgenic fertile barley lines expressing various codon-optimized *AMP* fusion genes either under constitutive or selected grain specific promoter were generated and analysed. Although immunolabeling using specific antibodies confirmed on protein level the accumulation of all of the designed AMPs in barley grain endosperm, it was not possible to detect recombinant pexiganan and human beta-defensin 2 using Western blot. Contrary to that, heterologous expression of human LL-37 in barley grains yielded up to 0.55 µg of recombinant peptide per gram of grain based on Western blot results. It was also shown that larger yields are achieved using a grain-specific than a constitutive promoter, that fusion of LL-37 to maltose-binding protein (MBP) increases its stability in desiccated grain and that cleavage of the LL-37 fusion protein using enterokinase results in efficient removal of the tags from recombinant products containing appropriate recognition sequence. Furthermore, the C- terminal KDEL extension in combination with N-terminal signal peptide resulted in accumulation of the product in endoplasmic-reticulum derived protein bodies that can be easily isolated for relatively low cost, which make this technology ideal for plant molecular farming with antimicrobial peptides.

Finally, the recombinant LL-37 exhibited biological activity against *E. coli* TOP 10 cells either after cleavage of the tag in the case of MBP or even in a fusion with a smaller 6xHis tag or KDEL tetrapeptide.

Abstrakt

Znepokojivý nárůst výskytu bakteriálních rezistentních kmenů představuje závažný problém v celosvětovém měřítku, který vyžaduje naléhavé řešení, aby nedošlo k tzv. post-antibiotické éře, kdy by rezistentní bakterie způsobující třeba i jinak "banální infekce" mohly mít opět na svědomí lidské životy. Antimikrobiální peptidy (AMPs) LL-37, beta-defensin 2 a pexiganan představují slibná činidla pro léčbu infekčních onemocnění způsobených rezistentními kmeny mikroorganismů, protože jsou aktivní vůči celé řadě patogenů včetně těch, jež si vyvinuly antibiotickou rezistenci. Navzdory jejich potenciálu je možnost praktického použití AMPs v medicíně limitována, a to především vysokou cenou spojenou s jejich produkcí. Z tohoto hlediska představuje využití rostlin pro jejich produkci velice slibnou strategii, která však vyžaduje řadu optimalizací. Ty jsou v zásadě spojeny s potenciální toxicitou AMPs vůči hostitelské rostlině, malým množstvím vyprodukovaného peptidu a případně také s problémy spojenými s izolací produktu a jeho biologickou aktivitou.

Ve snaze vyřešit výše uvedená omezení bylo vyselektováno několik fúzních peptidických či proteinových translokačních, stabilizačních a purifikačních sekvencí a byl testován jejich vliv na množství LL-37 přechodně akumulovaného v listech tabáku. Výsledky této analýzy poskytly hodnotná data, která byla zohledněna při návrhu konstruktů pro heterologní expresi LL-37 v ječmeni.

Následně byly metodou stabilní transformace připraveny a analyzovány fertilní linie ječmene exprimující buď pod konstitutivním, anebo vybraným zrnově specifickým promotorem různé varianty fúzních *AMP* genů podrobených kodonové optimalizaci pro ječmen. Přestože byla na proteinové úrovni metodou imunolokalizace s využitím specifických protilátek potvrzena přítomnost všech navržených AMPs v endospermu ječmenného zrna, nebylo možné metodou Western blotu detekovat rekombinantní pexiganan ani lidský beta-defensin 2. Naproti tomu Western blot analýza transgenních zrn ječmene exprimujících lidský LL-37 potvrdila akumulaci peptidu, která dosahovala hodnoty až 0.55 ug rekombinantního peptidu na gram zrna. Bylo prokázáno, že použití zrnově specifického promotoru je spojeno s dosažením větších výtěžků, než je tomu v případě konstitutivního promotoru, dále že fúze LL-37 k proteinu maltózu-vázajícímu (MBP) zvyšuje jeho stabilitu v desikovaném zrnu a že použití enterokinázy vede k účinnému odstranění značek z rekombinantních fúzních LL-37 produktů obsahujících příslušnou rozpoznávací sekvenci. Kromě toho přítomnost C-terminální KDEL sekvence v kombinaci s vhodným N-koncovým signálním peptidem vedla k akumulaci produktu v proteinových tělískách odvozených od endoplazmatického retikula, které lze snadno izolovat při relativně nízkých nákladech, což činí tuto technologii ideální pro produkci antimikrobiálních peptidů pomocí rostlinného molekulárního farmaření.

Závěrem byla prokázána biologická aktivita rekombinantního LL-37 vůči *E. coli* TOP 10 buňkám, a to buď po odštěpení fúzního proteinu v případě MBP, anebo dokonce ve fúzi s menší 6xHis kotvou nebo KDEL tetrapeptidem.

Aims of Work

1. A literature review on the topic of plant molecular farming with special emphasis on barley grain as a biotechnological tool, and also description of antimicrobial peptides and issues connected to their *in planta* expression.

2. Selection of antimicrobial peptides for recombinant production in barley.

3. Evaluation of effect of various fusion tags and localization signals on peptide accumulation.

4. Selection of promoters for gene transfer into barley.

5. Generation of transgenic barley lines expressing recombinant antimicrobial peptides and their molecular characterization.

6. Immunodetection of recombinant antimicrobial peptides in barley, their isolation and testing of antimicrobial activity.

1 Current state of knowledge

1.1 Antimicrobial peptides

Antimicrobial peptides (AMPs) are considered crucial effector molecules of the non-specific innate humoral immune response, as they act together with antimicrobial metabolites and stress related proteins during early stages of response to pathogens (Bent and Mackey, 2007). These low molecular mass peptides are evolutionary highly conserved and virtually ubiquitous in nature. Although variable in length, most of the AMPs are generally less than 50 amino acids long and can adopt a similar characteristic of forming an amphipathic structure. Most of the AMPs possess cationic character. Cationic AMPs are able to selectively recognize the prokaryotic cell membranes from the eukaryotic ones due to differences in their composition. The initial interaction between cationic AMPs and prokaryotic cell membranes is based on mutual electrostatic attraction that is typically followed by pore formation, which leads to one or more of many processes including micellization, membrane depolarization, cytoplasmic leakage, internalization of biocidal peptides or damage to intracellular macromolecule synthesis (Brogden *et al.*, 2005; Wimley *et al.*, 2010). As a result, the target cell death occurs within few minutes. This predominantly physical and unique mode of action of AMPs is associated to a very low risk of the emergence of resistant bacterial strains compared to classical antibiotics. Together with the fact that AMPs have been shown to display low cytotoxicity to mammalian cells, there is no wonder that these small peptides, also called peptide antibiotics, present a new generation of biocidal agents for various disease treatment in human and animals, especially in an era of increasing drug resistance in bacteria caused by extensive antibiotic use.

1.1.1 Human antimicrobial peptides

There are three major groups of host defense AMPs which can be found in human body, all of them playing a critical role in warding off invading microbial pathogens. These include cathelicidins, defensins and histidine-rich cationic linear histatins (MacKay *et al.*, 1984). In humans, the *CAMP* gene (Cathelicidine Anti-Microbial Peptide) represents the only one gene coding for the cathelicidin protein member that has been described so far. The gene product is known as the antimicrobial protein hCAP-18 that represents the precursor molecule from which the C-terminal antimicrobial domain known as LL-37 antimicrobial peptide can be released by proteinase 3 cleavage (Gudmundsson *et al.*, 1996). LL-37 is a small cationic 4.5 kDa peptide of 37 amino acids, overall charge +6, and an isoelectric point of 10.6. 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 (Kościuczuk *et al.*, 2012). LL-37 mediates a variety of biological functions. Its activation reflects the body's response to infectious and inflammatory stimuli or various injuries (Hancock and Diamond, 2000). Not only is the peptide an efficient antibacterial, antiviral and antifungal killer, but also mediates additional immune reactions such as wound healing, inflammatory responses or inhibition of tumorigenesis. Some of the examples of its potential practical application include the use of LL-37 as adjuvant in antibiotic-adjuvant pairing. Synergistic effects of LL-37 and antibiotics cause exert of therapeutic and bactericidal activity against target bacteria, including some of the MDR (multi-drug resistant) bacteria (e.g. *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*) that are of medical importance (Lin *et al.*, 2015; Nuding *et al.*, 2014). Quite recently, it has been shown that LL-37 might represent an Alzheimer's disease therapy candidate, as it is able to specifically bind to Aβ (a pathophysiological protein of Alzheimer's disease), and hence hinders its pathological accumulation (De Lorenzi *et al.*, 2017). Furthermore, LL-37 is being investigated in phase I clinical trial for its ability to induce anti-tumor immunity of melanoma patients with dermal metastases when applied in form of intra-tumoral injections (https://clinicaltrials.gov/ct2/show/NCT02225366, downloaded 28.5.2019). LL-37 might also be used in form of local application to the skin for the treatment of patients suffering from chronig leg ulcers,

as described in a study by Grönberg and co-workers (2014). In general, local application of antimicrobial peptides in form of creams, shampoos, lotions, and wound dressings seems to be the most advantageous over other possible forms of their administration, as they are susceptible to proteolysis by host peptidases, which may lead to loss or at least reduction of their effectivity (Rahnamaeian & Vilcinskas, 2015). Furthermore, when applied locally, AMPs might be delivered in higher concentrations to the affected area, as their absorption to the bloodstream and the associated side effects would be limited.

Defensins are evolutionary ancient, arginine-rich, small (4-6 kDa) cationic peptides found in various living organisms including humans and other mammals, fishes, birds, insects, fungi and plants (Liu *et al.*, 1997). Based on the composition of their primary structures and disulfide bridges formation are the human defensins divided into two distinct groups, namely the alpha-defensins and the beta-defensins, respectively (Winter and Wenghoefer, 2012). The expression of the human beta-defensin 2 (HBD2), also known as skin-antimicrobial peptide 1 is elevated at the site of skin injury and in chronic wounds, where they promote the expression of anti-inflammatory cytokines (Butmarc *et al.*, 2004). The urrogenital tract, in particular the male reproductive system, represent the major sites of the expression of most of the beta-defensins, whose levels varies depending on age, with the highest peak by the period of sexual maturity (Patil *et al.*, 2005). They are able to bind to the sperm plasma membrane and therefore to protect sperm cells against various pathogens in both the male and the female genital tract (Zhou *et al.*, 2004). Studies on antiviral activities of HBD2 showed that the peptide is active against severe human viral pathogens including human immunodeficiency virus, influenza A virus, and respiratory syncytial virus. These advantages could be taken for practical application of HBD2 to combat these viral infections. Moreover, human defensins may be also used as therapeutic adjuvants or vaccine carriers (Park *et al.*, 2018). Furthermore, it was demonstrated that β-defensins 2 might be utilized for second generation cancer vaccines (Biragyn, 2005). Another proposed utilization of human defensins also includes the “defensine vaccine” concept to protect human body against viral infections, which would be particularly useful during the flu season (Park *et al.*, 2018).

1.1.2 Pexiganan

Magainins represent a family of linear, cationic antimicrobial peptides that are composed of 21 to 27 amino acid residues. They are found in the skin of African clawed frog. So far, two types of magainins have been identified, magainin 1 and magainin 2, respectively. Magainin 2 represnet 23 amino acid residues long toroidal pore forming antimicrobial peptide (Imamura *et al.*, 2008), that was found to act as a broad spectrum antimicrobial agents. Due to high therapeutic potential of magainin 2, many of its synthetic analogues have been designed with the aim to maximize its broad spectrum activity. One of its best known analogues is Pexiganan (also known as MSI-78), which represents a 22 amino acid long AMP. In particular, its administration could be useful to overcome antibiotic resistance, as it possess broad antimicrobial spectra including MDR strains of bacteria while it retains low toxicity to mammalian cells. Pexiganan has undergone clinical trials for treatment of various more or less serious skin diseases. These incude diabetic foot ulcers, that might be treated in form of pexiganan acetate (1%), which would be locally applied as a cream to the affected area. However, although it was well tolerated by patients, the AMP has not been approved for marketing so far due to the fact that it is not more efficient than drugs already available on the market (Lipsky *et al.*, 2008). Besides that, pexiganan also entered clinical trials for the treatment of children with impetigo (https://adisinsight.springer.com/drugs/800002904, downloaded 3.6.2019). In addition, pexiganan could also serve in combination with approved β-lactam or glycylcycline antibiotics for the treatment of sepsis, as it possess synergistic activity with them (Cirioni *et al.*, 2018).

1.2 Plant molecular farming

The lack of large-scale cost-effective production technology of AMPs represents one of the main barriers for their everyday routine use in medical practice. One of the promising strategies includes the so-called plant molecular farming (PMF), where plant cells or tissues are used for expression and production of recombinant pharmaceutical proteins or peptides. Plants address advantages of mammalian or microbial cell culture methods but lack their pitfalls (Basaran and Rodriguez-Cerezo, 2008).

Developing barley grains possess efficient protein machinery with a rich mixture of different enzymes that enable correct folding of the heterologous protein. Moreover, a key advantage of seed cellular background is the presence of various types of protease inhibitors. Low protease content in barley grain together with a low content of water during dormancy allow long-term storage of heterologous proteins of interest at ambient temperature without loss of activity (Eskelin *et al.*, 2009; Patel *et al.*, 2000). Extraction and purification of the heterologous products are largely assisted by the fact that barley grain has relatively low content of secondary metabolites, is free of endotoxins, and has a simple protein profile. Moreover, barley holds certain agronomical advantages. There are powerful methods available for harvest, transport and storage of barley grains. Last but not least, domesticated diploid barley is a self-pollinating species. Thus, outcrossing with other non-transgenic plants is extremely rare (Ritala *et al.*, 2002). Additionally, barley holds the GRAS (generally regarded as safe) status from the U.S. Food and Drug Administration (FDA). Furthermore, after homozygous transgene fixation, it is also possible to grow the seeds on a field, and thus, increase the amount of recombinant product logarithmically.

For the purpose of PMF, achievement of high levels of recombinant products in desired plant tissues is crucial. Although transgene expression and target production can be increased by optimization of various parameters, choose of optimal promoter suitable for molecular farming hold the key to match the requirements for high protein accumulation. The use of promoters able to drive tissue-specific expression possesses several benefits over exploiting their ubiquitous counterparts. AMPs recombinantly produced in all parts of plant body may have negative pleiotropic effects on the vegetative growth (Hood *et al.,* 2003). With the use of strong grain-specific promoters, it might be possible to achieve higher accumulation levels of proteins in seeds compared to ubiquitous promoters. Since the barley endosperm is much larger than other tissues, and is also the major site for protein deposition, the largest group of promoters widely used in PMF are endosperm-specific. Most of the endosperm-specific promoters are derived from seed storage protein genes of barley or other cereals. Some of the examples of promoters that have been already tested for their ability to drive grain-specific expression in barley include rice *OsGLUB-1* promoter (Eskelin *et al.*, 2009; Patel *et al.*, 2000), barley endosperm specific hordein B-1 (*HOR2-4*) promoter (Joensuu *et al.*, 2006; Patel *et al.*, 2000), barley germination-specific aleurone α-amylase promoter (*α-AMY*) (Eskelin *et al.*, 2009), barley endogenous hordein D (*HOR3-1*) promoter (Erlendsson *et al.,* 2010), barley endosperm-specificβ-amylase prmoter (*β-AMY*) (Joensuu *et al.*, 2006), barley trypsin inhibitor (*TI*) prmoters (Joensuu *et al.*, 2006), barley *γ*-hordothionin promoter (Stahl *et al.*, 2009), wheat endosperm-specific high-molecular-weight glutenin Bx17 (HMW Bx17) promoter (Schünmann *et al.*, 2002), and the oat globulin 1 *(AsGLO1)* promoter (Hensel *et al.*, 2015).

1.2.1 Strategies to enhance stability of antimicrobial peptides

Heterologous expression of AMPs in plants for molecular farming has been limited. Technical restrictions leading to low product yield and instability still await innovative solutions. Researchers employ diverse strategies to achieve stability and accumulation. It has been proposed that one of the most crucial factor influencing not only *in vivo* stability of the recombinant peptide, but also the final yield is subcellular targeting of the product. The most commonly used strategy to enhance stability is inclusion of auxiliary signal sequences from source, host or closely related organisms to target the product to extracellular space (Bundó *et al.*, 2014; Coca *et al.*, 2006; Jan *et al.*, 2010). Most of the proteins or peptides lacking a signal peptide accumulate in the cytosol, generally resulting in low yields (Conrad and Fiedler, 1998). Next, targeting into native seed protein storage organelles (such as ER-derived protein bodies and *de novo* formed protein storage vacuoles) offer tremendous benefits in terms of product protection from degradation. Additionally, protein storage bodies aid in purification steps as well as post-harvest encapsulation. Next, fusion of a target AMP to a carrier protein tag is regarded as an effective strategy to stabilize the peptide, increase its accumulation, and protect the final product from proteolytic degradation. Moreover, protein fusions can mask the lethal effects of AMPs on host plant cells (Viana *et al.*, 2013). There are various tags widely used in heterologous expression of peptides. They can be grouped according to their common features and include easy to detect fusions, fusions to viral coat proteins, immunogenic protein partners, and purification-facilitating proteins (Viana *et al.*, 2013).

All these modifications might alter the activity and stability of the target peptide as well as the yields obtained from an expression system. On the other hand, stable transformation of certain hosts, specifically plants, is a laborious and slow process. Therefore, efforts in the last few decades have been dedicated to establishment of rapid screening platforms for evaluation of efficiency and yield of expression strategies before they are employed for generation of stably transformed plants. These screening platforms generally employ transient expression of the target peptide in a well-established host. Infiltration of intact leaf tissues with a suspension of *Agrobacterium tumefaciens* harboring an expression construct represent nowadays the most commonly used strategy for testing new constructs and generating valuable data. This labor-efficient, routine and cost-effective transient expression assay, with high transformation efficiencies, provide data with the analysis performed in several days without a need for selection pressure on leaf tissues. Majority of the analysis in literature have been performed using *N. benthamiana* leaves (Sparkes *et al.*, 2006).

1.3 Functional assessment of antimicrobial peptides expressed in plants

Function and antimicrobial activity of AMPs depend on three dimensional amphipathic structure of these peptides and their interaction with microbial membranes (Cruz *et al.*, 2014; Lee *et al.*, 2014). Post-translational modifications such as glycosylation, disulphide bond formation and folding are critical for maintaining AMP structure and biological activity. Although these modifications can be performed properly by plant cells, AMPs produced in heterologous plant-based systems might still have slight differences compared to their natural counterparts (Obembe *et al.*, 2011; Viana *et al.*, 2013). Similarly, synthetic AMPs might show different level of activity than that predicted *in silico*, after synthesis in a plant host. Furthermore, translational modifications such as fusions to secretion peptides or subcellular targeting signals, employed to boost expression in plant-based systems might alter the structure, function and stability of AMPs. Hence, evaluation of function and stability of heterologously produced AMPs are critical for intended uses in molecular farming and plant protection. Functional assessment of AMPs can be performed using *in vivo* or *in vitro* assays depending on the expression system employed, purpose of peptide production, the AMP synthesized and the target pathogen group. One of the most widely employed bioassays is inhibition tests performed *in vitro*. Total or crude protein extracts from transgenic plants expressing AMPs are used to inhibit microbial growth in diffusion assays on solid media or in liquid

bacterial suspension cultures (Jan *et al.*, 2010; Zakharchenko *et al.*, 2013a; 2013b). Number of viable microbial cells, concentration of AMP in the protein extract, culture conditions, duration of incubation, and various other parameters might affect the results obtained from these inhibition assays. Additionally, antimicrobial activity of contaminating endogenous proteins, peptides or metabolites from the host organism might also compromise the results. Hence, cautiously selected control reactions should be included alongside protein extracts from transgenic plants or cells.

Regarding the development of method for *in vitro* screening of *in planta* produced recombinant AMPs, consideration should be given to many steps, all of them being critical, as they determine the result of the analysis. These include selection of plant for antimicrobial screening and the tissue analysed, as various plants and their parts might express the target transgene at various level. Attention should be also given to selection of solvent system, time and temperature of extraction. As compounds presented in the plant crude protein extract may negatively affect analysis, thus give false negative results, further purification of target AMP and its enrichment in a given target volume of tested extract might be employed. When working with AMP fusion product, a special emphasis should be given to effective removal of the tag. After final preparation of the tested and control sample, selection of target microorganism for antimicrobial susceptibility testing, the size of inoculum, its quantification and preparation, type of antimicrobial screening method, time and temperaure of incubation and type of growth medium may influence the results of analysis. As there is no report describing *in vitro* functional assessment of plant produced LL-37 or its analogues, all of the aforementioned parameters had to be optimized in frame of this thesis to avoid overlooking of biological activity of LL-37 peptide.

2 Materials and Methods

All used material and methods are described in detail in the Ph.D. thesis.

3 Results

3.1 Assessment of plant-based strategies for LL-37 peptide production using transient expression in tobacco leaf tissues

In order to select the best strategy for *in planta* expression of LL-37, constructs for expressions of 10 different recombinant LL-37 (*rLL-37)* variants were prepared and used for infiltration of *N. benthamiana* leaves. The fused elements included *N*-terminal secretion signal sequence of cytokinin dehydrogenase 1 from maize (ZmCKX1sp), *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 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 (E) or the Factor Xa (X) recognition site were 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. Although RT-PCR analysis confirmed the accumulation of transgenic mRNA in all samples, Western blot analysis showed the presence of rLL-37 peptide only for 3 out of the 10 constructs. 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 rLL-37 peptide accumulation in plant tissue. Based on this observation, the three positive *rLL-37* peptide gene variants, *ZmCKX1sp_LL-37*, *ZmCKX1sp_LL-37_KDEL* and *ZmCKX1sp_6xHis_MBP_PRO_LL-37_KDEL* were selected for the heterologous expression in barley. Moreover, two additional variants *ZmCKX1sp_$(GGGGS)_2$_6xHis_$(GGGGS)_2$_E_LL-37* and *OsCht1sp_6xHis_E_LL-37* consisting of gene coding for ER transit peptide *ZmCKX1sp* or *OsCht1sp* (rice *chitinase 1*) enterokinase *recognition sequence* and *6xHis* tag, which was in one case flanked by gene for a flexible linker sequence *$(GGGGS)_2$* to ensure effective separation of the domains were designed.

3.2 Selection promoters driving endosperm preferrable expression for subsequent molecular farming purposes in barley

Based on results obtained through Genevestigator platform, 5 barley endogenous genes with endosperm-preferred expression were selected as the most promising candidates to drive tissue-specific accumulation of rLL-37 in grains. The considered candidate genes included: *B Hor* (B1 hordein), *D Hor* (D hordein), *Hinb* (hordoindoline b), *CI2* (chymotrypsin inhibitor 2), and *TI* (trypsin inhibitor). Expressions of the genes were compared using qPCR analysis. As shown in Figure 1B, the barley B1 hordein gene promoter revealed the strongest expression levels of corresponding endogenous gene in later developmental stages of wild-type barley spikes. Furthermore, the *B Hor* gene promoter was used to direct stable overexpression of cytokinin oxidase/cytokinin dehydrogenase 9 gene (*HvCKX9*), where the tissue specific gene delivery was found in T2 homozygous barley plants using qPCR assay. The relative transcript levels of this cytokinin metabolizing gene were determined in barley endosperm, embryo, aleurone, and seed coat tissues. Raised expression of *HvCKX9* gene was observed in all transgenic samples tested. However, the predominant overexpression of transgene was observed in endosperm tissue, with the relative transcript level being approximately 32 500 times increased compared to non-transgenic control

plants. Thereby, the *B Hor* promoter was selected to drive controlled endosperm-specific accumulation of most of the AMP variants expressed in barley in scope of this thesis. In addition, promoter of trypsin inhibitor gene was also selected for driving the endosperm specific accumulation of recombinant pexiganan (*rPEX)*, as the expression profile of its corresponding endogenous gene showed strong and strictly endospem-specific accumulation (see Figure 1A), but it seems to be predominantly active in later stages of barley seed growth and develoment (Fig. 1B). Finally, based on an extensive search in the literature, the oat globulin promoter was also exploited to drive tissue specific accumulation of the recombinant human beta-defensin 2 (*rHBD2*) gene.

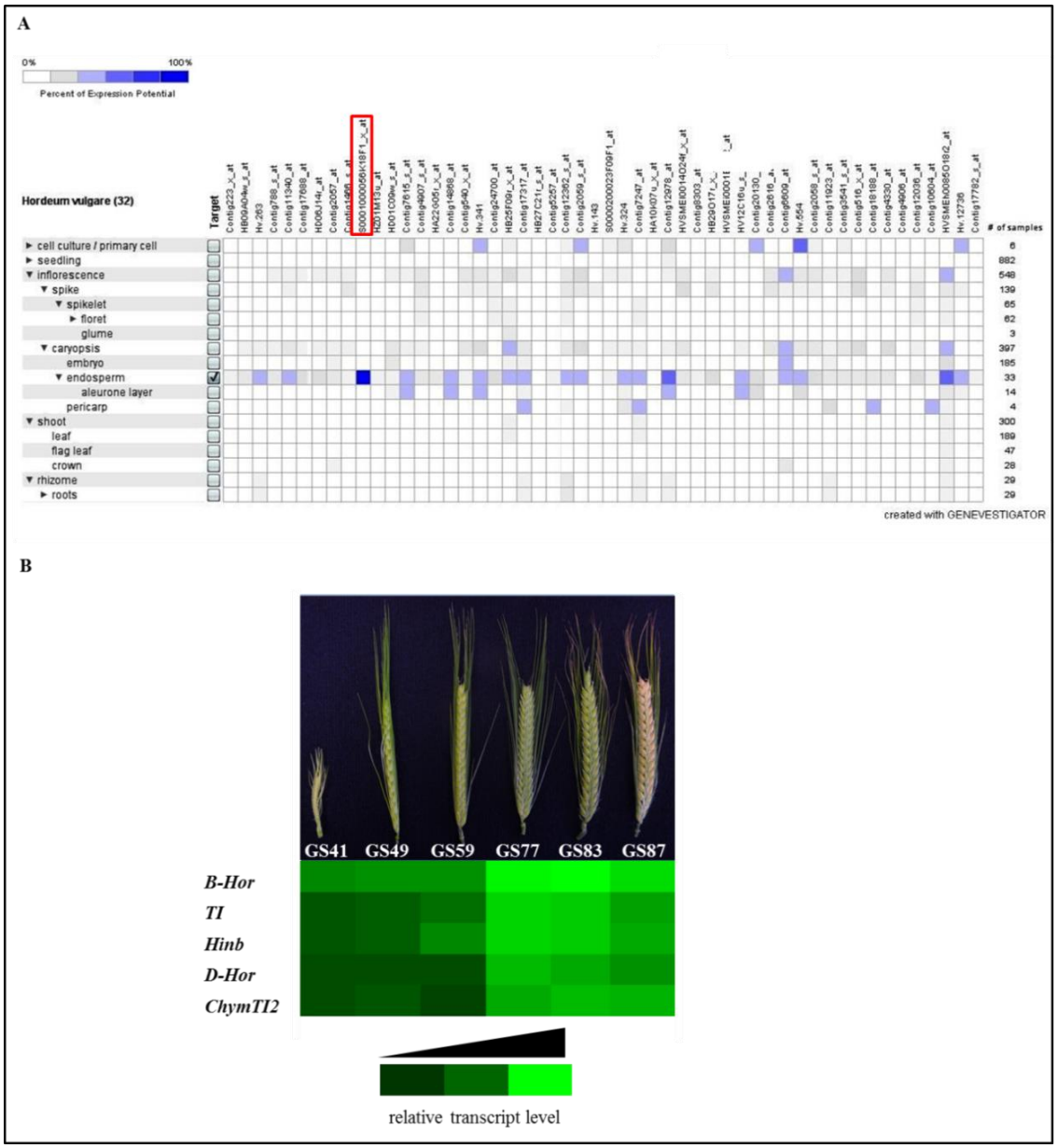

Figure 1: Results from GENEVESTIGATOR search (A) and temporal expression profiles of selected barley genes (B). B1 hordein (*B Hor*), trypsin inhibitor (*TI;* contig enclosed in red rectangle), hordoindoline b (*Hinb*), D hordein (*D Hor*) and chymotrypsin inhibitor 2 (*CI2*) genes were shown to be preferably expressed in endosperm (A). Temporal control of expression driven by their native promoters was analysed as the relative transcript levels by qPCR using ΔΔCt relative quantification method corrected by an efficiency factor (B).

3.3 Generation of stable transgenic barley lines expressing rAMPs

In total, 10 various transformation vectors for either endosperm specific (B-HORp, TIp, AsGLO1p lines) or ubiquitous (UBIp lines) *rAMPs* expression in barley were prepared. These included:

B-HORp::ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL;
B-HORp::ZmCKX1sp_LL-37_KDEL;
B-HORp::ZmCKX1sp_LL-37;
B-HORp::ZmCKX1sp_$(GGGGS)_2$_6xHis_$(GGGGS)_2$_E_LL-37;
B-HORp::OsCht11sp_6xHis_E_LL-37;
UBIp::ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL;
UBIp::ZmCKX1sp_LL-37_KDEL;
UBIp::ZmCKX1sp_$(GGGGS)_2$_6xHis_$(GGGGS)_2$_E_LL-37;
TIp::PEX_KDEL;
AsGLO1p::8xHis_E_HBD2.

Subsequently, 162 independent T0 stable transgenic barley lines expressing genes coding for various rAMPs were prepared using agroinfection of wounded immature barley embryos. The total number of inoculated embryos reached approximately 3 300 and the overall transformation efficiency expressed as the number of independently transformed T0 plants per 3 300 inoculated embryos was about 5%. For further propagation to subsequent generations, only diploid T0 plants harboring either single or low copy number of T-DNA inserts were selected. Neither ubiquitous nor grain specific accumulation of LL-37 (Figure 2) resulted in abnormal growth characteristics and morphology of the transgenic barley lines and the same was true for single copy T-DNA lines expressing *rPEX* gene. Contrary to that, single copy *rHBD2* expressing lines showed extended phenotype that might be attributed to the type of AMP expressed and its properties that negatively affected barley plant growth, as this phenomenon was observed in all of the 3 independent transgenic T1 and T2 transgenic lines examined.

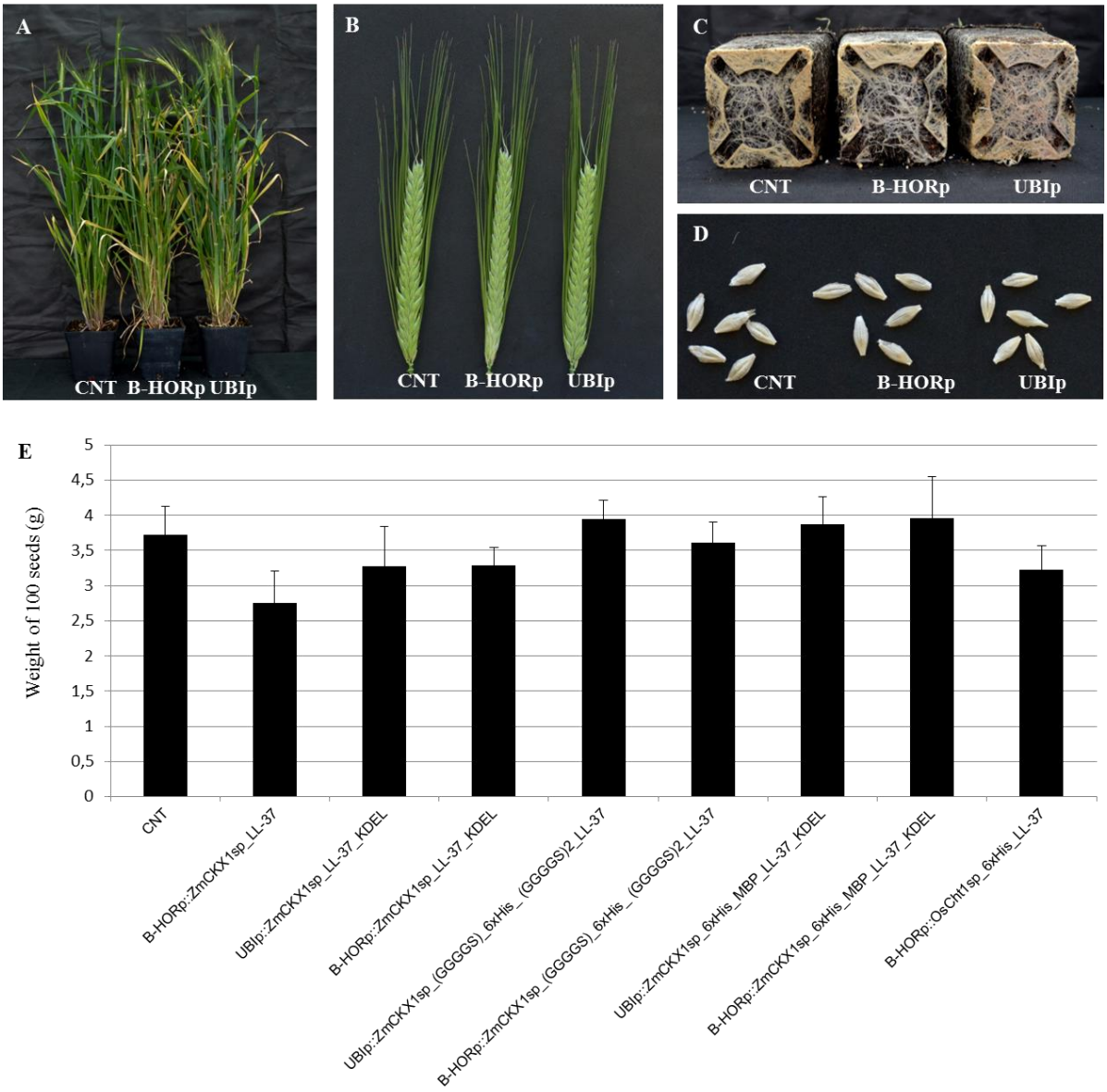

Figure 2. Comparison of phenotype of T1 transgenic barley plants expressing rLL-37 under grain specific B1 hordein gene promoter (*B-HORp*) or the maize ubiquitin gene promoter (*UBIp*) to untransformed tissue culture regenerated barley plants (CNT). Representative photos of the aerial part (A), spikes (B), roots (C), and mature grains (D) are displayed. (E) Yield parameters of transgenic lines, average weights of 100 mature grains of transgenic barley plants carrying the individual transgenes are indicated. Error bars represent the standard deviation of 3 independent lines per transformation event and at least 3 plants per line (with the exception of line *B-HORp::ZmCKX1sp_LL-37_KDEL*, in which 10 plants per only 1 line were analysed).

3.4 Molecular analysis of regenerated barley plants

Based on PCR analysis it was demonstrated that individual transgenes were stably integrated into the barley genome and inherited. Most of the transgenic barley plants were diploid (92%). The transgene copy number was determined by segregation analysis showing that less than 50% of the transformants were single copy T-DNA insertion lines with segregation ratio being 3:1. Furthermore, genomic DNA of randomly selected T0 lines was also subjected to Southern blotting using *hpt* gene-specific DIG-labelled DNA hybridization probes. As expected, obtained results were in concordance, as plants of 3:1 Mendelian segregation were confirmed to be a single copy T-DNA

insertion lines using Southern blot, suggesting that both of the experimental approaches are applicable for this type of analysis.

To check wheter the transgene copy number is proportional to the gene expression level, cDNA isolates from late milk endosperm grains of selected T0 plants expressing *ZmCKX1sp_(GGGGS)$_2$_6xHis_(GGGGS)$_2$_E_LL-37* gene under *B-HORp* were analysed by means of qPCR assay. However, no clear correlation between transgene expression on RNA level and transgene copy number was observed, as some of the multiple copy gene insertion lines showed one of the weakest and other ones the strongest expression.

Next, to see whether all *rLL-37* variants were successfully transcribed in roots, leaves and grains of the studied transgenic barley lines, a set of randomly chosen T1 plants of *B-HORp* and *UBIp* lines was subjected to RT-PCR analysis using primers specific to the coding regions of individual transgenes. The *rLL-37* gene transcripts in the barley *B-HORp* lines were detected only in grains, but not in roots or leaves. Opposite to that, the *UBIp* lines showed the presence presence of *LL-37* amplicons in all analysed tissues. Hence, the functionality of the used promoters, as well as the transgenic expression of *rLL-37* genes, was clearly confirmed.

Analogously, the relative levels of transgene transcripts in selected T1 *PEX* and T2 *HBD2* expressing lines were determined, not only to confirm functionality of the expression vectors under study, but also to gain deeper insight into *TIp* and *AsGLO1p* activities. To do so, qPCR analysis was performed. All of the analysed lines showed highest product accumulation in seeds compared to roots or leaves. However, the *AsGLO1p* seems to be more appropriate for biotechnology applications using barley seeds as biofactories than the *TIp*, as it drives much stronger and target more effectively the expression of desired gene into this organ.

3.5 Detection of rAMPs in barley grain sections by immunolabeling

To test the presence of rAMPs in transgenic barley lines and obtain more detailed information about their distribution/accumulation patterns, sectioned mature barley seeds were subjected to immunolabelling with specific antibodies.

The presence of recombinant pexiganan and human beta-defensin 2 in confirmatory *in situ* immunolabeling tests resulted in the blue-violet coloration of endosperm of transgenic barley lines (Figure 3). *PEX* expressing lines showed non-specific staining of embryos, whereas no such coloration was observed for *HBD2* expressing lines. Differences may be attributed to different specificities of the antibodies used for the analysis.

Regarding the visualisation of rLL-37 in barley grains, all transgenic lines showed purple staining of endosperm reflecting the presence of rLL-37 peptide, while the endosperm of control lines remained unstained. Much greater intensity in staining was achieved in the presence of the endoplasmic reticulum (ER) retention signal, the KDEL sequence at C-terminus. Staining in the grain sections that expressed the peptide under the control of *B-HOR* promoter was more intense than in the case of *UBI* promoter (Fig. 4). Furthermore, as demonstrated in Figure 4B, accumulation pattern of rLL-37 was stably inherited over at least 3 successive generations.

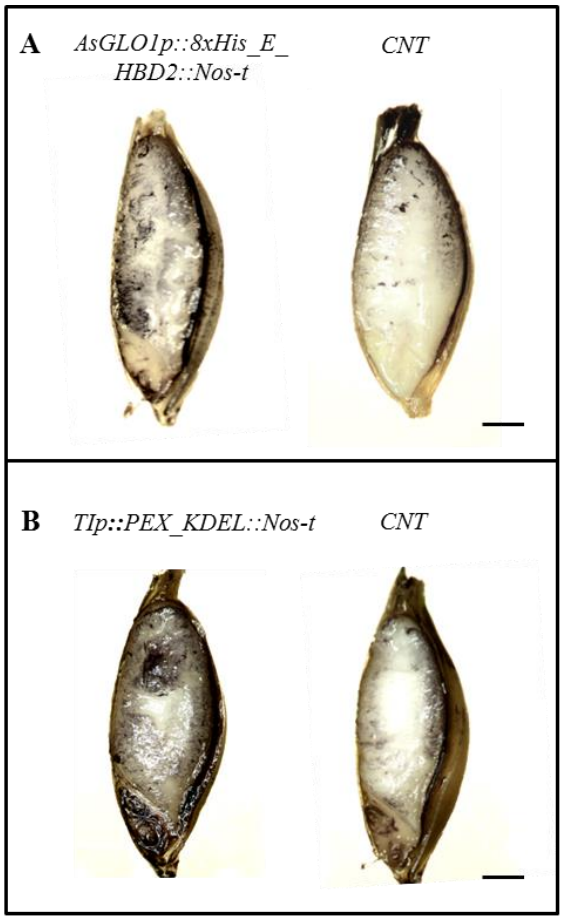

Figure 3. Representative figures of colorimetric detection of recombinant human beta-defensin 2 (HBD2, A) and pexiganan (PEX, B) antimicrobial peptide in desiccated seeds (BBCH 99) of T1 barley lines carrying the indicated transgenes. CNT, control non-transgenic tissue culture regenerated plant. Scale bars correspond to 1 mm.

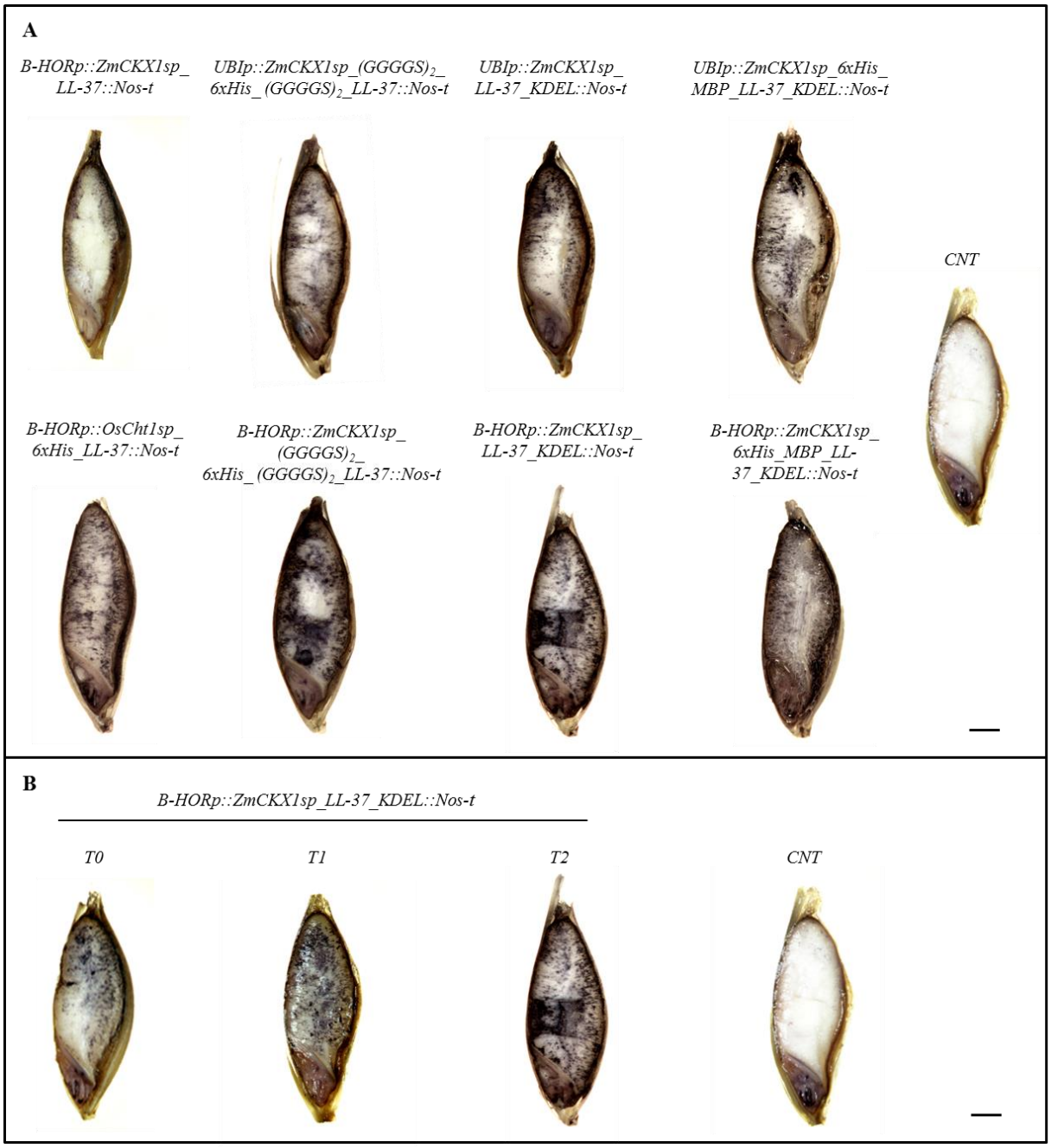

Figure 4: Representative figures of colorimetric detection of different variants of recombinant LL-37 (rLL-37) peptide in desiccated seeds (BBCH 99) of T2 barley lines carrying the indicated transgenes (A) and confirmation of stable integration and translation of integrated transgenes through three successive generations (T0, T1, T2; B). CNT, control non-transgenic tissue culture regenerated plant. Scale bars correspond to 1 mm.

3.6 Detection of rAMPs in protein extracts by Western blot

Next, protein extracts from chosen extraction and purification steps were subjected to Western blot analysis to see whether the transgene transcripts were properly translated in individual barley tissues. Unfortunately, none of the extract prepared from *rPEX* and *rHBD2* expressing lines, purified or not, as well as the protein bodies' enriched fractions gave positive result when analysed by Western blot. This is in contrary to the findings obtained from immunolabeling analysis, that gave clear evidence about the presence of rPEX and rHBD2 AMPs in transgenic barley grains.

Differences might be attributed to lower sensitivityof Western blot analysis where significantly higher content of the target peptide would be required to produce a detectable signal.

Whereas no specific signals were detected in crude protein extracts from leaves, roots and grains of the transgenic lines expressing rLL-37, Western blot analysis revealed the presence of rLL-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 (Fig. 5). When purifying rLL-37 peptides containing 6xHis and MBP epitope tags, the elimination of contaminating proteins by affinity chromatography greatly enriched the content of the corresponding rLL-37 peptide. Furthermore, when the epitope tags used for purification were cleaved off by enterokinase, the released rLL-37 peptides well matched the expected size (Fig. 5A). Purifications of ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL protein on either Co^{2+}-IDA-agarose or amylose affinity column provided comparable results. Non-tagged variants were not recovered from crude extracts in any case. Chimeric rLL-37 peptides extended with endoplasmic reticulum (ER) entry signal sequence ZmCKX1sp at *N*-terminus and *C*-terminal KDEL tetrapeptide ER retention sequence were successfully recovered from protein bodies' enriched fraction (Fig. 5AB) and accordingly no product was extracted from protein bodies when lacking the KDEL sequence. As shown in Table 1, content and stability of rLL-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 *B-HORp* provided much higher levels of rLL-37 peptide in grains than the expression driven by *UBIp*. Although analysis of *UBIp* lines provided clear evidence of the presence of rLL-37 peptide also in roots and leaves of transgenic plants (Fig. 5B), 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 rLL-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 Western blot 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). 6xHis tag (Co^{2+}-IDA) purified protein fractions prepared from barley lines expressing *B-HORp*::*ZmCKX1sp_(GGGGS)$_2$_6xHis_(GGGGS)$_2$_LL-37* analysed by MALDI-TOF-MS showed a peptide of monoisotopic mass of 7161 Da, which was absent in the samples from control plants (analysis performed by prof. Mgr. Marek Šebela, Ph.D., Faculty of Science, Palacký University Olomouc). The detected mass exactly corresponds to that of the rLL-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.

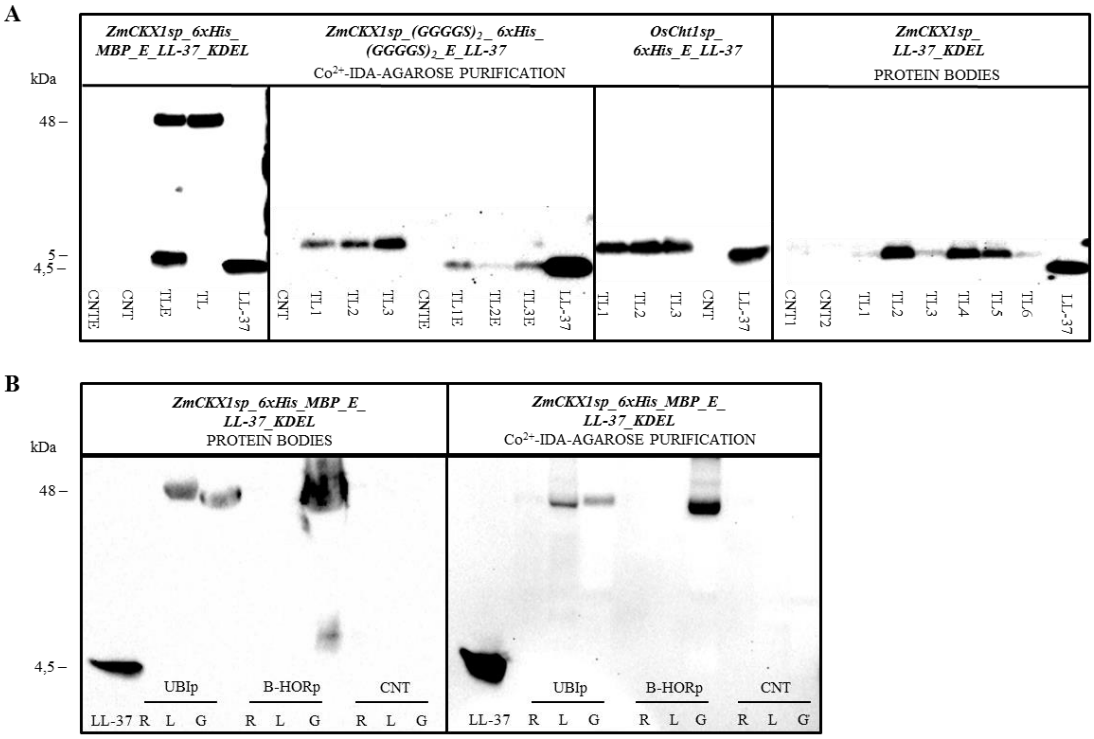

Figure 5. Western blot analysis of recombinant human LL-37 (rLL-37) in protein extracts of several T1 transgenic lines (T) and control non transgenic tissue culture regenerated plants (CNT). (A) Detection of rLL-37 in protein bodies or purified protein fractions from late milk endosperm seeds (BBCH 77) 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 marked as (CNE) and (TLnE), respectively; 7 ng of synthetic LL-37 served as positive control. (B) Detection of rLL-37 in purified protein fractions prepared from roots (R), leaves (L) and late milk endosperm grains (G) of transgenic lines expressing the indicated transgenes under grain specific promoter of the barley B1 hordein gene (*B-HORp*), or the maize ubiquitin gene promoter (*UBIp*); 7 ng of synthetic LL-37 served as positive control.

Table 1. Accumulation levels of LL-37 in grains of T1 generation of transgenic barley lines as estimated by Western blot analysis of Co^{2+}-IDA purified protein extracts and protein body enriched fractions. BBCH scale 77 and 99 correspond to late milk endosperm seeds and desiccated seeds, respectively. *B-HORp*, transgenic lines expressing *rLL-37* under grain specific B1 hordein gene promoter; *UBIp*, transgenic lines expressing *rLL-37* under maize ubiquitin gene promoter. Displayed are the mean accumulation values with standard deviations of estimated amounts of rLL-37 peptide from 3 independent lines per transformation event and at least 3 plants per line (with the exception of *B-HORp::ZmCKX1sp_LL-37_KDEL*, where only 1 line was analysed). X, transgenic lines were not prepared; N.D. product not determined.

***rLL-37* gene**	**Co^{2+}-IDA-agarose purification** (µg of rLL-37 per kg of grains)				**Isolation of protein bodies** (µg of rLL-37 per kg of grains)			
	Late milk endosperm (BBCH 77)		Desiccated grain (BBCH99)		Late milk endosperm (BBCH 77)		Desiccated grain (BBCH 99)	
	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

3.7 Examination of antimicrobial activity of recombinant human LL-37 products

Since the antimicrobial activity of the LL-37 peptide has been documented elsewhere, only a simple test was conducted whether the rLL-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 (Gallo *et al.*, 2010). As shown in Fig. 6, 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 *B-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_E_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_$(GGGGS)_2$_E_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_E_LL-37* transgene, which peptide product is 6xHis_E_LL-37, resulted in 40% bacterial growth inhibition. To sum up, the rLL-37 peptide produced in barley plants including short tag elongated versions on either N- or C- termini are biologically active.

Protein extracts of barley *rPEX* and *rHBD2* expressing lines were not subjected to analysis of antimicrobial activity due to low concentration of AMPs in the protein extracts prepared (based on Western blot analysis results).

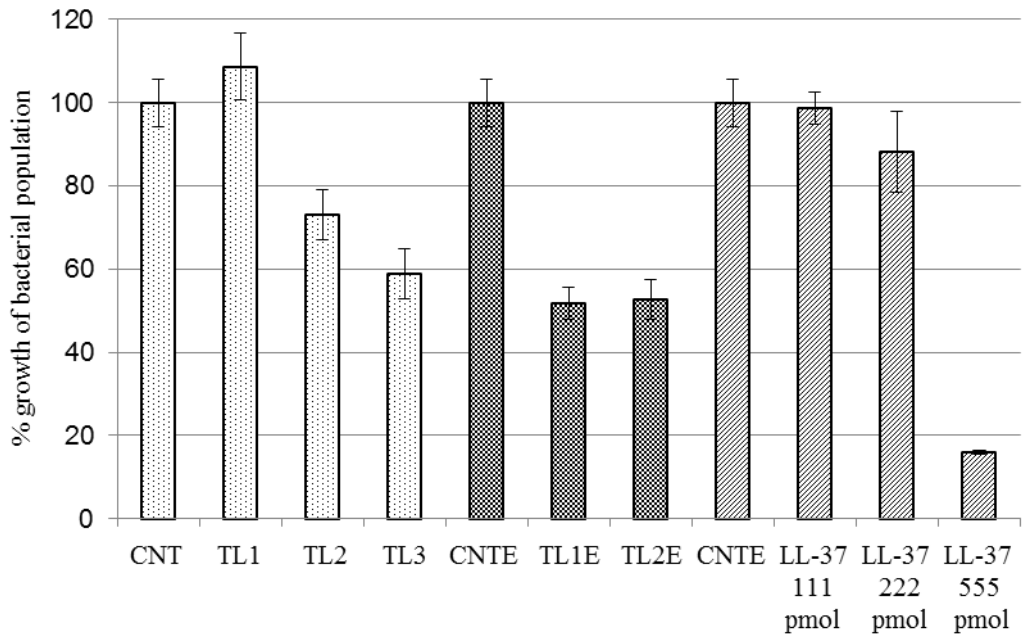

Figure 6. *In vitro* antibacterial activity of the synthetic LL-37 peptide and purified proteins prepared from late milk endosperm grains (BBCH 77) of T2 generation of transgenic lines. The biological activity was tested against *E. coli* TOP10, which have been mixed with 10 µL of Co^{2+}-IDA agarose purified fraction containing between 1 to 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. CNT, purified protein extracts from control non-transgenic tissue culture regenerated plants; TL1, purified protein extracts from lines expressing *ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL* transgene; TL2, *ZmCKX1sp_$(GGGGS)_2$_6xHis_$(GGGGS)_2$_E_LL-37* transgene; TL3, *OsCht1sp_6xHis_E_LL-37* transgene. CNTE, enterokinase digested purified protein extracts from control plants; TL1E, enterokinase digested purified protein extracts from lines expressing *ZmCKX1sp_6xHis_MBP_E_LL-37_KDEL* transgene; TL2E, *ZmCKX1sp_$(GGGGS)_2$_6xHis_$(GGGGS)_2$_E_LL-37* transgene. Error bars represent standard error of results of two independent experiments.

4 Discussion

Economic feasibility of AMP production in plants is mainly dependent on development of a proper business strategy to match the anticipated benefit with biopharming; i.e., reduced cost of goods. One of the basic and most difficult practical considerations is the choice of final pharmaceutical product itself. In the present study, human LL-37, pexiganan and human beta-defensin 2 were selected out of the pool of existing AMPs based on intensive search in literature and subsequently expressed in transgenic barley and eventually also in tobacco leaves. Two of the main attributes for their selection taken under consideration were as follows: structure of the target peptide should not be cyclic due to possible difficulties connected to its proper folding, and the selected peptide should be applicable in commercial sector, e.g. cosmetic or pharmaceutical industry. Below there will be first discussed possible applications of the selected AMPs in modern medicine.

Although plant based producion of AMPs provides tremendous benefits over other production systems, especially in terms of production costs, certain technical limitations still remain. These are in general connected to the quantity and quality of the produced peptide, that reflects not only the amount of expressed transgenic RNA, but also the efficiacy of its translation, stability and biological activity of the produced peptide and its recovery. Additionaly, the expressed AMP might exhibit a certain level of phytotoxicity, that may cause abnormal undesirable plant phenotype. Accordingly, many parameters needf to be optimized on both RNA and protein levels to boost the yield of the final product. As its has shown to be problematic to express peptides of less than 50 amino acids in cells of cereal plants, possibly due to the silencing mechanisms or rapid degradation of the peptide (Takagi *et al.*, 2008; Takagi *et al.*, 2010; Yasuda *et al.*, 2005), several plant expression vectors for the production of LL-37 fused to various functional protein or peptide domains were designed and screened for evaluation of yield of expression before they were employed for preparation of stably transgenic barley plants. For the screening purposes served agroinfiltration of tobacco leaves. Results of this analysis clearly showed that N- terminal fusion of LL-37 to sorting signal sequence seems to be essential to be the transgene expressible and subsequently detectable on protein level, probably due to fast degradation of those products lacking it. Furthermore, it has been shown previously that an addition of KDEL tetrapeptide is essential to target *in planta* produced recombinant proteins to membrane-bound organelles, either the so-called protein bodies (PB-I) (Bundó *et al.*, 2014; Company *et al.*, 2014), or to protein storage vacuoles (PB-II) (Arcalis *et al.*, 2004) that are responsible for so-called bioencapsulation of the product. 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 (Stöger *et al.*, 2005). Effect of the ER retention sequence on the higher productivity of recombinant LL-37 has been confirmed in transient tobacco assay where 3-fold higher accumulation was observed in the case of construct bearing the KDEL sequence. Accordingly, all of the *LL-37* gene variants designed for the expression in transgenic barley included N- and eventually also C- terminal KDEL targeting signal to direct the AMP accumulation to certain subcellular compartments. 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 (Bell *et al.*, 2013). 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.

Selection of promoter sequence is no less important for proper transgene expression in transgenic plant, as it holds the key to match the requirements for high expression levels. Accordingly, it was necessary to select promoters suitable for *AMPs* expression in barley. In numerous studies, expression of *AMP* was driven by the constitutive *UBIp*. Advantages of constitutive *AMP* expression

in barley may lay in high product yield, as a recombinant product could be theoretically recovered from all of its tissues. On the other hand, ubiquitous expression and subsequent accumulation of an AMP might negatively affect biological functions in a host plant as stated in introduction to this thesis. With this respect, organ-specific regulation of gene expression can help reduce this risk. Regarding barley, grains represent the most suitable target tissue for recombinant protein accumulation, as their natural properties should enable not only robust protein accumulation, but also its long-term storage and simple downstream processing. Since an endosperm forms approximately three quarters of a barley grain, thus represent its largest morphological part, it is advantageous to direct the expression to this tissue. In addition, the barley grain endosperm is used by ORF genetics to produce biorisk-free growth factors (epidermal and stem cell growth factors) and cytokines for use in medical research, skin care and stem cell technology, that are available under the trademarks ISOkine ™ and Bioeffect ™ (http://www.orfgenetics.com/, downloaded 20/06/2019). The company built their large greenhouses in Iceland, where most of the primary energy supply is derived from renewable or cheap sources such as geothermal energy, hence price of energy in Iceland is among the cheapest in the world. In scope of this study, several candidate promoters driving endosperm-preferred expression were selected from a barley genetic background using Genevestigator screening tool. As a next step, strength of the expression driven by the candidates during development of a barley grain was compared, as application of strong promoters able to drive expression in later phases of endosperm tissue development seems to be beneficial for the purpose of plant molecular farming. The promoter of the barley B1 hordein gene followed by the trypsin inhibitor gene promoter showed the best results in this respect, thus both of them were employed in *AMPs* expression strategies. Since the functionality of the *B-HORp* has been verified previously by generation of transgenic lines overexpressing the barley *CKX9* gene (Holásková, 2012), plus its properties were deeply characterized in scope of this thesis using the *CKX9* overexpressors, it was incorporated in most of the vectors for grain-specific accumulation of AMPs.

As the *in situ* immunolabeling analysis confirmed, although at various extend, the presence of all of the recombinant products under study in mature barley grains, next goal was their isolation. Unfortunately, neither the Western blot analysis of protein extracts of *rPEX* nor *rHBD2* expressing lines gave positive result. This failure might attributed to the absence of peptide localization sequence at the N-terminus, which was probably reflected in low stability of the product and subsequently its very low level in mature barley grains. These results are in concordance to those obtained from agroinfiltration of tobacco leaf tissues with constructs for transient expression of human cathelicidin variants lacking N-terminal translocation signal. Besides, negative results may be ascribed to inappropriate extraction techniques used that resulted in low extraction efficiency. In contrast to that, 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. The amount of produced rLL-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 (Bundó *et al.*, 2014). Cabanos *et al.* (2013) 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 (Lee *et al.*, 2011; Zeitler *et al.*, 2013). 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.

Analysing 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. Although the MBP fusion was expected to boost the amount of recombinant protein product in all of the stages of barley grain development, total accumulation of MBP fused to LL-37 was lower than this of free peptide in milky endosperm, but fused peptide was slightly more abundant in desiccated grain than the non-fused rLL-37 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 rLL-37 peptide. In both cases, a purified peptide fraction was obtained. Higher yields of the peptide, however, were achieved by isolating the ER-derived protein bodies (into which LL-37 was deposited when expressed with the secretion signal peptide and the C-terminal tetrapeptide KDEL) by simple gradient centrifugation in sucrose solution according to Bundó *et al.* (2014) and thereby, purification time and costs were significantly reduced compared to chromatographic methods. Comparison of product amount of rLL-37 obtained using isolation of protein bodies versus affinity purification has shown that the loss of recovery yields of rLL-37 fused to MBP represented about 85% in late milk endosperm grains and about 99% in desiccated grains when using chromatography column separation. Hence, targeting into storage organelles seems to be much more beneficial for subsequent industrial or pharmaceutical large scale production of AMPs. In addition, a study by Takagi and co-workers (2010) revealed, that cereal seed ER-derived protein body may represent an effective tool for oral delivery of peptide and protein therapeutics. Authors of this study demonstrated, that a tolerogen for the control of pollen allergy when localized in rice seed derived PB-I showed increased protection from the enzymatic digestion in simulated gastric fluid.

The LL-37 peptide's antibacterial activity is conformation-dependent. It can adopt either disordered or helical structure which represents its biologically active form. Transition from disordered structure to alpha-helix positively correlates with the antimicrobial activity of the peptide. At micromolar concentration, the LL-37 peptide exists in water in a disordered structure. Formation of alpha-helix is dependent on LL-37 peptide concentration (the higher the concentration, the greater extent of helical conformation), on the pH value and on the presence of anions such as HCO_3^-, CO_3^{2-}, or SO_4^{2-} (Gallo *et al.*, 2010; Johansson *et al.*, 1998). Accordingly, all of the purified rLL-37 either cleaved by enterokinase or not, were buffer exchanged for NH_4HCO_3 in order to test their ability to inhibit bacterial growth. The LL-37 exhibited biological activity against *E. coli* TOP 10 cells either after cleavage of the tag in the case of MBP or even in a fusion with a smaller 6xHis tag or KDEL tetrapeptide. These results are in concordance with a study by Mičúchová (2017), who showed similar results in terms of retaining biological activity of LL-37 fused to C-terminal KDEL extension. In addition, Coca and co-workers (2006) and also Bundó and co-workes (2014) have demostrated, that rice plant produced cecropin A peptide designed for retention in endoplasmic reticulum exhibited antifungal and antibacterial activity, respectively. On the other hand, the antimicrobial activity of the LL_KDEL product was unsuccessfully tested by Janechová (2018), who subjected the peptide for the antibacterial activity assay against a gram-positive bacterium *Micrococcus luteus*, even though that the group by Kim *et al.* (2009) observed clear growth inhibition zone against

M. luteus when placing recombinant hCAP18/LL37 produced in its active form by *Pichia pastoris* on top of the test media poured by tested bacterial culture (Kim *et al.*, 2009). Observed variations in rLL-37 activity might be attributed to different methods used for functional assessment of this peptide, as it has been shown previously, that antimicrobial efficiency of AMPs strongly depends on assay parameters including the applied medium in which the microbes are treated (Farkas *et al.*, 2018). Together these data indicate, that although rLL-37 antimicrobial properties were confirmed using *E. coli* TOP10 cells, there is no guarantee that *in planta* produced recombinant LL-37 that has been shown to be effective against one bacterial strain using one test assay will also be active against another pathogen using different assay indicating that it is necessary to optimize the antimicrobial activity assay for each case.

5 Conclusions

In conclusion, human cathelicidin LL-37, human beta-defensin 2 and *Xenopus laevis* derived pexiganan were selected from diverse natural and synthetic peptides with antimicrobial activities for production in barley, as they may represent valuable products for innovative applications in medical or cosmetic industry. Although heterologous expression of AMPs using plant-based systems is regarded as a key to the bottleneck for their large-scale cost-efficient production, many technical limitations that must be surmounted still remain. To respond accurately to this challenge, several modifications of *LL-37* gene were designed and evaluated for yield using agroinfiltration of tobacco leaf tissues. Based on results obtained from the screening technology used, stable transgenic barley lines expressing various codon-optimized *AMP* fusion genes either under constitutive or selected grain specific promoter were generated and analysed. Most of the transgenic plants showed similar growth rate dynamics and morphological characteristics as the control tissue culture regenerated lines. Immunolabeling using specific antibodies confirmed the accumulation of individual AMPs, although to various extents, in barley grain endosperm, and stable expression and storage of the product at room temperature over several years in ceed cellular background was confirmed by analysis of 3 successive generations of *B-HORp::ZmCKX1sp_LL-37_KDEL::Nos-t* lines. It was demonstrated that human cathelicidin can be produced by molecular farming in barley either as peptide *sensu stricto* just with the ER retention tetrapeptide or in the fusion with MBP that increases its stability in desiccated grain and cleavage of the LL-37 fusion protein using enterokinase results in efficient removal of the tags from recombinant products containing DDDDK pentapeptide. It was also shown, that the grain-specific expression provides higher product yields in grains than the constitutive one and the largest accumulation occurs in the stage of milky endosperm. The C- terminal KDEL extension in combinantion with N-terminal signal peptide sequence of *Zea mays* cytokinin oxidase/dehydrogenase 1 resulted in accumulation of the product in ER- derived protein bodies (PB-I), as confirmed by subcellular fractionalization. Furthermore, isolation of the recombinant LL-37 by preparation of a protein body enriched fraction resulted in higher product recovery compared to the protein isolation using chromatographic methods. This attribute along with the fact that a presence of the KDEL sequence should not have negative impact on LL-37 antimicrobial properties, as demonstrated by antibacterial activity assay using *E. coli* TOP 10 cell, and together with the fact that bioencapsulation should be resposible for at least partial protecion of recombinanat proteins or peptides from enzymatic digestion by host proteases, make this technology ideal for production of peptide therapeutics. To sum up, results of this work show that biologically active AMPs can be produced in barley using various viable approaches, which might be in future applicable for their commercial production (Fig. 7).

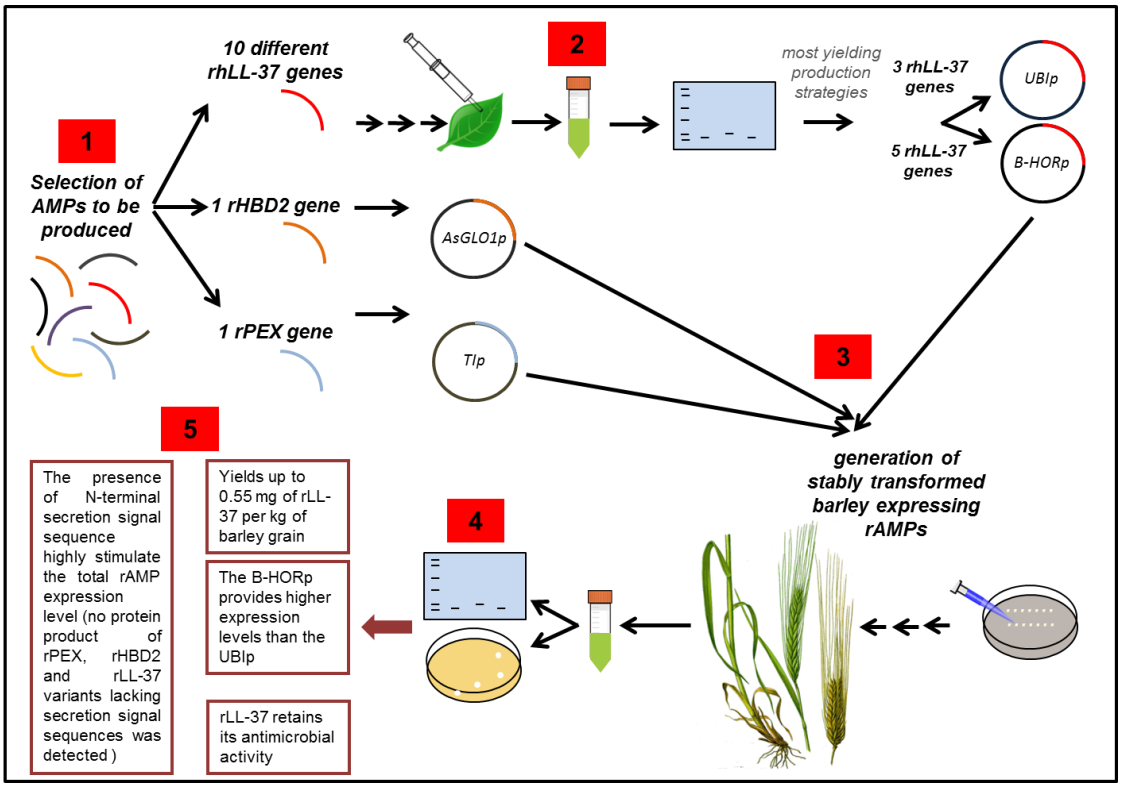

Figure 7. Flow diagram highlighting the key points of this work. (1) Some of the antimicrobial peptides (AMPs) that might be applicable in therapeutic or cosmetic industry were selected from large varieties of AMPs. (2) Effects of different fusion partners on AMP production using transient expression in tobacco leaves was assessed. (3) Selected chimeric genes were cloned under chosen promoters and used for the generation of transgenic barley lines expressing *AMPs*. (4) The effect of different expression strategies on accumulation levels and antimicrobial activity was assessed. (5) Results of this work provide important data for plant molecular farming with low molecular weight peptides.

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7 Curriculum vitae

Personal data

Name and surname:	Edita Holásková
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Education

2012 – present
Palacký University in Olomouc
Ph.D. study in Biochemistry

- Research project: Generation and analysis of new transgenic barley lines expressing recombinant antimicrobial peptides.

2010 – 2012
Palacký University in Olomouc
MSc. Biochemistry

- Research project: Preparation of transgenic barley with cytokinin altered level in grains.

2007 – 2010
Palacký University in Olomouc
BSc. Biochemistry

- Research project: Monitoring of the influence of purine derivates on root development of *Arabidopsis thaliana*.

Employment

2012 – present
Student research assistant
Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University in Olomouc

Research fellowships

July – August 2015
Biotechnology Research Center, Shanxi Academy of Agricultural Sciences, Taiyuan, China
prof. Yi Sun

- The goal was to get practical experience in 2 various methods for genetic manipulation of maize, that were developed at the local research center.
- Oral presentation of results of my Ph.D. research project.

January – March 2014
Freie Universität, Berlin, Bundesrepublik Deutschland

- The goal was to analyse HIPP transformants of *Arabidopsis thaliana* at the protein level.
- Oral presentation of results of my Ph.D. research project.

Teaching
Basic laboratory techniques for biochemists (KBC/LABT)
Advanced biochemical methods (KBC/BAM)
Summer biotechnology project 1 (KBC/LBP1)
Supervisior of 1 master and 3 bachelor students

Oral conference presentation
2017
Holásková E, Galuszka P, Frébort I. Transgenic barley as a bioreactor for the production of recombinant cationic α-helical antimicrobial peptides. *Green for Good IV*, Olomouc, Czech Republic.

Poster conference presentations
2015
Holásková E, Öz MT, Mičúchová A, Galuszka P. Plant-based production of antimicrobial peptides with therapeutic potential. *Green for Good III*, Olomouc, Czech Republic.

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Holásková E, Öz MT, Galuszka P, Frébort I. Molecular Farming: Plants as Vehicles for the Production of Antimicrobial Peptides. *12th PhD Student Conference of Plant Experimental Biology*, Olomouc, Czech Republic.

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Holásková E, Öz MT, Galuszka P, Frébort I. (2014): Molecular Farming: Plants as Vehicle for the Production of Antimicrobial Peptides. *The first Conference of the International Society for Plant Molecular Farming*, Berlin, Bundesrepublik Deutschland.

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Holásková E, Öz MT, Galuszka P, Frébort I. (2013): Biopharming as a tool for production of antimicrobial peptides. *2nd Conference of Cereal Biotechnology and Breeding*, Budapest, Hungary.

Publications
Holásková E, Galuszka P, Mičúchová A, Šebela M, Öz MT, Frébort I. 2018. Molecular farming in barley: development of a novel production platform to produce human antimicrobial peptide LL-37. *Biotechnology Journal* 13(6): e1700628.

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Mrízová K, Holásková E, Öz MT, Jiskrová E, Frébort I, Galuszka P. 2014. Transgenic barley: a prospective tool for biotechnology and agriculture. *Biotechnology Advances* 32, 137-147.

Awards
2013: Awarded by director of the CRH for excellence in category "Contracted research" for verification of production of antimicrobial peptides in the grain of transgenic barley.

Skills
English, German, Driving licence, Computer skills

Others
Member of the Academic Senate of the Faculty of Science of Palacký Univ