DOI: 10.29252/nbr.5.3.262

سیستمهای بیان پروتئین مبتنی بر گیاه برای تولید پروتئین و پیتید ضدمیکروبی

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دریافت: ۱۳۹۷/۰۵/۱۵ / ویرایش: ۱۳۹۷/۰۶/۲۰ / پذیرش: ۱۳۹۷/۰۶/۳۱ / انتشار: ۱۳۹۷/۰۹/۳۰

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چکیده. گیاهان یک سیستم بیانی برای تولید پروتئینهای هترولوگ، به خصوص پروتئینهای نوترکیب درمانی هستند. سالهای اخیر، اکثر پروتئینهای نوترکیب در باکتریها یا سلولهای پستاندار تولید میشوند. اگر چه گاهی سلولهای حشرات، مخمر و قارچ برای تولید پروتئینهای نوترکیب استفاده می-شود. محصول سیستم گیاهی می تواند سالم، اقتصادی و راحت باشد و این امکان وجود دارد به طور گسترده در کشاورزی و صنایع، به ویژه در علوم زیستی و صنعت داروسازی استفاده شود. گیاهان سیستمهای بیانی پروتئینی مناسب در تولید با کیفیت، هزینه کم و تغییرات پساترجمهای صحیح هستند. استفاده از گیاه تراریخته در تولید واکسنها، آنتی بادیها و پروتئینهای دارویی در سالهای اخیر تبدیل به یک نقطه عطف در مهندسی ژنتیک گیاهی شده است. استراتژی-های کارآمد برای تولید پروتئینهای نوترکیب اهمیت بیشتری را به دست می آورند، به عنوان برنامههای کاربردی و مهم نیاز به مقدار زیادی از پروتئینهای نوتر کیب با کیفیت بالا برای استفاده در صنعت است. در این مقاله، مشکلات رایج در تولید پروتئینهای نوترکیب و پیتید ضدمیکرویی در سیستمهای بیانی یر و تئین مبتنی بر گیاه مورد بررسی قرار گرفته و استراتژیهای راه حل آنها مورد بحث قرار گرفته است.

واژههای کلیدی. آنتی بادی، پروتئین های دارویی، پروتئین های نوترکیب، گیاهان تراریخت

Plant-based expression systems for protein and antimicrobial peptide production

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Received: 06.08.2018 / Revised: 11.09.2018 / Accepted: 22.09.2018 / Published:21.12.2018

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Abstract. Molecular farming technology offers a unique advantage that almost any protein can be produced economically and safely under very controlled conditions. Besides traditional production systems, such as bacteria, yeasts, insects and mammal cell lines, plants can now be used to produce eukaryotic recombinant proteins, especially therapeutic ones. Their advantages as hosts for protein production include correct post-translational modifications, lowcost maintenance and no risk of contamination by human pathogens. The system is widely applied in agriculture and industry, especially in life science and pharmaceutical industry. The application of transgenic plants in the production of vaccines, antibodies and pharmaceutical proteins has been playing a key role in plant genetic engineering in recent years. The production of recombinant proteins plays a critical role in the production of high amounts of high-quality proteins. In this review, common problems in the production of recombinant proteins and antimicrobial peptides in plant-based expression systems are discussed and strategies for their solution are suggested. Viral vector-mediated transient gene expression in plants enables rapid production of pharmaceutical proteins such as vaccine antigens and antibodies. To conclude, plant-based systems have the potential to bring unique efficacy-enhancing features to increase the utility and effectiveness of vaccines and therapeutics.

Keywords. antibodies, pharmaceutical proteins, therapeutic proteins, transgenic plant

INTRODUCTION

Among various expression systems such as bacteria, yeast, mammalian cells and insects, plants

are becoming an attractive system for the of recombinant pharmaceuticals, industrial proteins, monoclonal

antibodies and vaccine antigens, due to a large number of advantages including safe usage, rapid scale-up, long-term storage and low-production cost (Daniell et al., 2001, 2009b; Fischer and Emans, 2000; Fischer et al., 2003, 2004, 2013; Horn et al., 2004; Howard and Hood, 2005; Klimyuk et al., 2008; Ma et al., 2003, 2005; Rybicki, 2009; Schmidt, 2004 Thomas et al., 2011). In addition, plant cells have the ability to perform posttranslational modifications (PTM) of proteins, which are typical of eukaryotic organisms (Gomord and Faye, 2004; Mett et al., 2008; Vitale and Pedrazzini 2005). PTM affects the proteins activities and functions such as folding, stability, solubility as well as dynamic interactions with other molecules. The major type of PTM for plant-made recombinant proteins is glycosylation (Stulemeijer and Joosten, 2008; Webster and Thomas, 2012). PTM is an action following translation for additional covalent modifications with changing tertiary and quaternary structures of proteins.

A wide variety of promoters have been used for protein expression in plants, including constitutive, inducible and tissue-specific, native and synthetic promoters (Egelkrout *et al.*, 2012). The most

widely-used promoter is the cauliflower mosaic virus 35S promoter (CaMV35S), which promotes high-level of gene expression through the transcriptional terminator of the *Agrobacterium* nopaline synthase gene (*nos*) (Barampuram and Zhang, 2011; Lee *et al.*, 2008). Several molecular events, such as gene transcription, mRNA translation and protein accumulation, affect overall success of protein expression (Table 1).

The recombinant proteins accumulation in plant system depends on several factors including a choice of suitable host tissue and subcellular location, understanding the nature of the foreign proteins and determine their possible effect on the host plant as well as the post-translational modifications and the degree of protein purification (Egelkrout *et al.*, 2012; Fischer *et al.*, 2004; Schiermeyer *et al.*, 2004).

Recently the phenomenon of recombinant protein expression and its corresponding impact have sparked a heated debate. Although contested by many, the matter of plant system is highly beneficial. Therefore, there are several options for protein expression in plant systems (Table 2) that will be discussed in this review.

Table 1. Summary of factors affecting gene transcription, mRNA translation and protein accumulation Source: Modified from Egelkrout *et al.* (2012)

Factors affecting transcription rate	Factors affecting translation rate	
Promoter selection	Post-transcriptional gene silencing	
Transcription factor binding (TFB) sites	Codon usage	
Upstream regulatory sequences (URS)	mRNA stability	
Intron-mediated sequences	Recruitment and retention of translation-ralated protein	
Transcriptional gene silencing (TGS)	Factors affecting protein accumulation	
Pre-mRNA stability	Protein stabilisation	
Transgene trait stacking	Protein localization	
The impact of the site of integration	Protein modification	
The number of gene copies	Cellular milieu	

Table 2. List of required options for plant expression systems (Egelkrout et al., 2012)

options for plant expression system			
Choice of host plant			
dicot or monocot			
food or non-food			
Type of transformation method			
biological (viral, bacterial)			
physical (biolistic, electroporation)			
Expression parameters			
stable or transient			
constitutive or tissue-specific			
The intracellular location			
cytoplasm or apoplast			
Organelle			
Plastid			

Host plants

Protein expression platforms have been developed for a variety of host systems including seed crops (e.g., corn, canola, soybeans and rice) leafy crops (e.g., tobacco, alfalfa and lettuce), plant cell cultures (e.g., tobacco, carrot and rice), hairy root cultures and aquatic plants (e.g., *Lemna minor*), in addition to moss and green algae (Wilken and Nikolov, 2012). One of the major plant systems used for the production of recombinant proteins is tobacco. Because of high leaf biomass yields, it can be cropped several times a year (Basaran and Rodriguez-Cerezo, 2008; Twyman *et al.*, 2003; Twyman, 2004; Wilken and Nikolov, 2012).

Nicotiana tabacum L. (Tobacco)

The genus Nicotiana belongs to the family Solanaceae, which includes 76 species, originating in North America, South America, Australia and Africa (Chase et al., 2003; Knapp et al., 2004). Nicotiana tabacum is an amphidiploid/allotetraploid species with 48 chromosomes, generated from hybridisation between N. sylvestris and either N. tomentosiformis Goodspeed or N. otophpra Grisebach, all of which originate in South America (Kitamura et al., 2001; Lewis, 2011). Nicotiana tabacum, as a model plant in research, provides many practical advantages for large-scale production of recombinant proteins, including high biomass yield, low maintenance and high speed production (Twyman, 2004). It is the non-feed crop which carries a reduced risk of transgenic material contaminating food chains and contains a hight amount of nicotine and other alkaloids that are necessary to be removed (Stoger et al., 2005; Twyman et al., 2003; Twyman, 2004). Ma et al. (1995, 1998) reported the first successful expression of the secretory monoclonal antibody in transgenic N. tabacum. The two tobacco cell lines, BY-2 (bright yellow 2) and NT-1 (N. tabacum 1), are readily available for Agrobacterium-mediated genetic transformation and have been optimised for rapid growth (Doran, 2013).

Nicotiana benthamiana Domin

The non-cultivated tobacco species, *Nicotiana benthamiana*, is a unique Australian native plant that may have resulted from hybridisation between *N. suaveolens* and *N. debneyi* (Goodspeed, 1954; Goodin *et al.*, 2008). It is an amphidiploid species with 38 chromosomes (Goodspeed, 1954) that used as a host in the production of monoclonal antibodies and vaccines. It is a suitable host species to produce recombinant proteins using viral vectors such as tobacco mosaic virus (TMV) (Twyman, 2004; Yang *et al.*, 2004). It can be transformed with high efficiency and maintained easily, due to its short stature, short regeneration time and high seed production. It is also useful for the transient expression of genes (Goodin *et al.*, 2008).

Transformation methods and expression systems

Genetic transformation can be achieved by physical or biological methods. Physical methods are usually used to transfer non-viral vectors by using a gene gun (biolistics) and chemical polyethylene glycol (PEG) transformation or electroporation of protoplasts (Barampuram and Zhang, 2011; Rao et al., 2009). The biological method of plant cell genetic transformation by Agrobacterium involves the transfer and integration of a large tumor-inducing (Ti) or rhizogenic (Ri) plasmid resident in Agrobacterium into the plant nuclear genome. Although several species of Agrobacterium are known, two species are predominantly transformation: used for Agrobacterium tumefaciens (T-DNA transfer for generation of transgenic plants) and Agrobacterium rhizogenes (Ri-plasmid transfers necessary DNA to generate transgenic hairy roots) (Gelvin, 2003; Gleba et al., 2014; Rao et al., 2009).

The production of recombinant proteins in plants, also known as molecular farming, is classified into two expression systems, i.e., stable and transient expressions. Stable expression involves integration of foreign DNA into the plant genome and transfer to the next generations (Sharma *et al.*, 2005). The storage organs such as seeds have been used for the stable expression of proteins of interest (Scheller and Conrad, 2004). The chloroplast and nuclear stable transformations require more than two month, on the one hand, and several months, on the other. (Patiño-Rodríguez *et al.*, 2013).

Transient expression systems include *Agrobacterium*-mediated and viral-based transformation, wherein the foreign gene is not integrated in the genome and being expressed only for a few days after being introduced into the plant cell/tissue.

The strategy relies on A. tumefaciens, which contains transgenes; the bacteria are infiltrated directly into the organs of interest by injection or by vacuum chamber (agroinfiltration) (Klimyuk et al., 2014; Leckie and Stewart, 2011; Leuzinger et al., 2013; Vaghchhipawala et al., 2011). Transient expression is generally used to verify the activity of the expression constructs and generates small amounts of recombinant protein for functional analyses (Twyman et al., 2003). Many results have been obtained so far with viral backbones such as those of the RNA viruses, Tobacco mosaic virus (TMV), Potato virus X (PVX) and Cowpea mosaic virus (CPMV) (Gleba et al., 2014; Marillonnet et al., 2005). Transient expression, using viral vectors, is one of the best and most rapid methods to obtain a high yield of protein expression (Egelkrout et al., 2012).

Expression systems

The production of recombinant proteins in plants, also known as molecular farming, is classified into two expression systems includeing stable and transient expressions.

Stable expression

Stable expression involves the production of transgenic plants or transgenic cell cultures. The foreign DNA is integrated into the plant genome and is transferred to the following generations (Sharma *et al.*, 2005). The storage organs of crop plants such as seeds have been used for the stable expression of the proteins of interest (Fischer *et al.*, 2004).

Transient expression using viral vectors

Transient viral-based systems are amongst the best available systems and have the advantage of serving as a rapid method for large-scale production of proteins such as antibodies and vaccine antigens (Gleba et al., 2007; Pogue et al., 2002). The viral vector system is based on two strategies: 'full virus' vectors used to express long polypeptides (at least 140 amino acids) and 'deconstructed' virus vectors, which rely on A. tumefaciens to deliver DNA copies of one or more viral RNA replicons to plant cells (Gleba et al., 2007; Gleba and Giritch, 2012). One of the main systems of deconstructed virus vectors is the 'magnICON®' from ICON genetics, based on the tobacco mosaic virus (TMV), which, instead of supplying whole RNA or linear DNA, it is divided in different modules captured between the left and right border of T-plasmid of A. tumefaciens (Gleba et al., 2004; Gleba et al., 2014; Marillonnet et al., 2004).

The gene of interest is cloned in a 3' vector module. Various 5' modules are then used for targeting the expressed protein to different parts of the plant cell (cytosol, chloroplast or apoplast). An integrase module is used for the recombination of 3' and 5' modules in planta. These modules recombine within the plant cell, the resulting DNA transcribed and the recombination sites (integrase) are spliced out in order to create an RNA replicon to produce the desired protein (Marillonnet et al., 2004). Three A. tumefaciens cultures deliver different vector modules containing different viral components, targeting sequences. These pro-vector 5' and 3' modules assemble within the plant cell in the form of a functional viral vector (Marillonnet et al., 2004). After being delivered by agroinfiltration, they are assembled inside the cell with the help of a sitespecific recombinase to create an RNA replicon to produce the desired protein (Fig. 1).

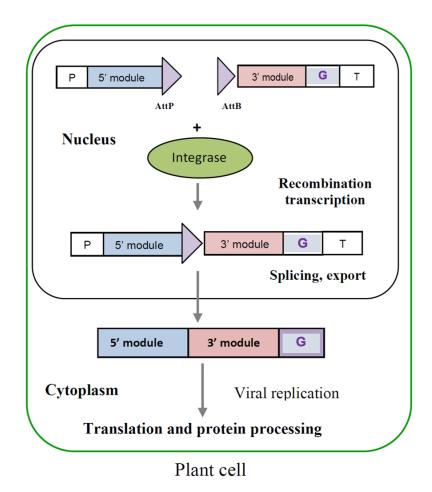


Fig. 1. Schematic showing the assembly of the three viral vector modules and production of protein. The 5' and 3' modules with integrase in the plant cell allow recombining via the AttP and AttB sites. The intron is then spliced out. Viral replication occurs and translation and interest protein processing occurs. P: ACT2 promoter, T: *nos* terminator, G: Gene of interest. Source: Modified from Marillonnet *et al.* (2004).

The advantage of this system is the high-yield production of the desired protein because of viral amplification (Marillonnet et al., 2004). Targeting the proteins into different subcellular compartments can alter protein stability (Gils et al., 2005) e.g., the improvement of stability by chloroplasts (Doran, 2006). The green florescence protein (GFP) was expressed in different subcellular compartments of N. benthamiana using this system and produced the recombinant protein at up to 80% of total soluble protein (Marillonnet et al., 2004, 2005). A considerable amount of research has been published on the use of the magnICON® system (Gleba et al., 2004, 2005, 2007; Gils et al., 2005; Giritch et al., 2006; Huang et al., 2010; Marillonnet et al., 2004, 2005; Niknejad et al., 2016; Santi et al., 2006, 2008; Werner et al., 2011).

Subcellular location in a plant system for protein accumulation

The targeting of foreign proteins to the most appropriate subcellular compartment is important for protein accumulation, stability, folding and post-translational modification (Twyman, 2004). The choice depends on the structural characteristics of the protein and the use of appropriate signals (Benchabane *et al.*, 2008; Faye *et al.*, 2005). Several subcellular compartments including the cytosol, endoplasmic reticulum (ER), apoplast, vacuole and chloroplast have been considered (Benchabane *et al.*, 2008; Ma *et al.*, 2003).

Cytosol

Recombinant proteins are retained in the cytosol when no targeting signals are used. Several studies have revealed that recombinant proteins remain stable within the cytosol (De Jaeger *et al.*, 1999; Michaud *et al.*, 1998; Marusic *et al.*, 2007). However, the cytosol may not be the best location

for several reasons: high levels of protease and ubiquitin activity, the negative redox potential that may affect the correct folding of proteins via formation of disulphide bonds, and a lack of important co- and post-translational modifications, such as glycosylation, which may affect folding, assembly and the tertiary structural stability (Benchabane *et al.*, 2008; Faye *et al.*, 2005).

Endoplasmic Reticulum (ER)

Targeting proteins to the ER can lead to high yields of recombinant proteins *in planta* (Conrad and Fiedler, 1998; Wandelt *et al.*, 1992). Entry of recombinant proteins into the endoplasmic reticulum (ER) can be retained by adding (SEK/H/K)DEL retention sequences to the C-terminal (Benchabane *et al.*, 2008; Michaud *et al.*, 1998; Mainieri *et al.*, 2004), in most cases a (K/H) DEL sequence (Benchabane *et al.*, 2008; Ma *et al.*, 2003). The presence of chaperones in the ER helps to di-sulphide bonds formation and correct folding of the recombinant protein (Benchabane *et al.*, 2008; Faye *et al.*, 2005).

Apoplast

Proteins can be targeted into the apoplast via the secretory pathway (Benchabane *et al.*, 2008). The plant apoplast includes the cell wall matrix and intercellular spaces in a wide range of physiological functions (Witzel *et al.*, 2011). The lack of KDEL in endosperm-expressed protein results in targeting to the apoplast, where the recombinant protein mostly accumulates in the space under the cell wall (Christou *et al.*, 2004).

Vacuole

The vacuole is one of the alternative destinations for recombinant proteins produced in plants (Benchabane *et al.*, 2008). Additionally, it serves several key roles in planta e.g., control of cell turgidity, levels of macromolecules, accumulation of toxic secondary metabolites and storage of highenergy compounds in vegetative tissues (Benchabane *et al.*, 2008; Twyman, 2004).

Chloroplast

The chloroplast is considered to be one of the cellular destinations in a plant expression system through the addition of an appropriate targeting signal peptide (Hyunjong *et al.*, 2006). The recombinant proteins may be moved to the chloroplast. Chloroplast transformation offers uniform expression rates, a high copy number of the transformed genes (due to hundreds of plastids in a cell), co-expression of multiple genes from the

same construct, minimal transgene escape in the environment due to the maternal inheritance of chloroplast DNA and minimal gene silencing (Daniell *et al.*, 2004).

Post-translational modifications, such multimerisation and disulphide bridge formation, can occur in the stroma (Daniell, 2006; Parachin et al., 2012). Thus, the chloroplast can be considered to be a suitable compartment with no need for the cell secretory pathway or modifications such as although, glycosylation, some endogenous proteases can affect the stability and accumulation of recombinant proteins (Adam and Clarke, 2002). However, this may not be relevant issue for proteins expressed at very high yield (Daniell, 2006).

Plant systems for heterologous production of antimicrobial peptide

Anti-microbial peptides or proteins (AMPs) identified from prokaryotes and eukaryotes are usually less than 100 amino acid residues long and often have a high frequency of positively charged amino acids (Silva et al., 2011). They are strongly cationic (pI: 8.9-10.7) having a net charge of 2+ to 7+, mainly due to the presence of arginine, lysine and histidine residues, heat-stable (100°C, 15 minutes), and additionally, they may have 50% hydrophobic amino acids and do not have any lysing/killing effects on eukaryotic cells (Hancock and Chapple 1999; Li et al., 2012). AMPs have shown to serve as an important mechanism of natural resistance and also as a metabolically rapid inexpensive first line defence against pathogens (Brokaert et al., 1997; Egorov et al., 2005).

The antimicrobial activity of such peptides was observed to involve ionic interactions between their basic residues and the negatively charged bacterial membrane constituents, such as phospholipid head groups (Jenssen *et al.*, 2006; Melo *et al.*, 2009; Shai, 2002). On the other hand, the outermost layer of the eukaryotic membranes is composed of lipids with no net charge and zwitterionic head groups positioned within the inner layers; membrane-binding is controlled by hydrophobic rather than ionic interactions (Lee *et al.*, 2011a).

During the past 15 years, a wide range of AMPs have been shown to play essential roles in plant defense systems. Their sizes range from 2 to 9 kDa and many of them have a cysteine-rich structure with 6 or 8 cysteine residues involved in disulphide bridges; e.g., thionins, defensins, non-specific lipid transfer proteins (nsLTPs), hevein - and knottin-like peptides, MBP1, IbAMP and snakins (Broekaert *et*

al., 1997; Benko-Iseppon et al., 2010; Egorov et al., 2005; García-Olmedo et al., 1998; Nawrot et al., 2014; Odintsova et al., 2009). Some of the cysteine-rich AMPs, such as defensins, thionins and LTPs, are observed in wheat plants (Egorov et al.,

2005). The hevein-like peptide with a 10 cysteine motif isolated from *Triticum kiharae* has shown antifungal and antibacterial activity (Dubovskii *et al.*, 2011; Odintsova *et al.*, 2009).

Table 3. AMPs heterologous produced in Nicotiana tabacum

AMP transformed	Source	Objective	Resistance against	References
LFchimera	chimerical peptide	Production peptide and antimicrobial activity	Escherichia coli, Staphylococcus aureus, Erwinia amylovora, Ralstonia solanacearum	Chahardoli <i>et al.</i> (2018)
protegrin-1 (PG-1)	Synthesized peptide	Production peptide and antimicrobial activity	Klebsiella pneumoniae, Staphylococcus aureus, Escherichia coli, Mycobacterium bovis, Candida albicans	Patiño-Rodríguez et al. (2013)
Dermaseptin Lysozyme C4V3	Phyllomedusa sauvagii, Gallus gallus, Synthetic	Production and isolation of the peptide and vaccine	Anti-HIV activity, induction of mammalian immune response	Rubio-Infante <i>et al.</i> (2012)
EtMIC2	Eimeria tenella	Peptide production	Eimeria tenella (chicken coccidiosis)	Sathish <i>et al.</i> (2011)
Retrocyclin- 101 and Protegrin-1	Artificial AMP based on rhesus monkey (Macaca mulatta) circular minidefensins	Chloroplast genome transformation aiming peptide production	Erwinia carotovora, tobacco mosaic virus	Lee et al. (2011b)
NmDEF02	Nicotiana megalosiphon	Proof of concept for crop improvement	Phytophthora parasitica var. nicotianae, Peronospora hyoscyami f.sp. tabacina, Alternaria solani, Phytophthora infestans	Portieles <i>et al</i> . (2010)
Trichokonins	Trichoderma pseudokoningii	Proof of concept for crop improvement	Tobacco mosaic virus	Luo et al. (2010)
Pal and Cpl-1	Phages infecting S.pneumoniae	Chloroplast genome transformation aiming peptide production	Streptococcus pneumoniae	Oey et al. (2009)
MsrA2 (N-methionine-dermaseptin B1)	Phyllomedusa sauvagei and Phyllomedusa bicolor	Gene stacking for crop improvement	Fusarium solani, Fusarium oxysporum, Alternaria alternata, Botrytis cinerea, Sclerotinia sclerotiorum, Pythium aphanidermatum, Pectobacterium carotovorum	Yevtushenko & Misra (2007)

Antimicrobial peptides/proteins (AMPs) are unique biologically active molecules that comprise defense systems against numerous pathogens such as bacteria, fungi, parasites and/or viruses. Heterologous expression systems, including bacteria and fungi as host cells, have been used for the production of AMPs with different sizes, folds and complexities (Parachin et al., 2012). Plant systems have also been used as suitable sources for the production of AMPs and can be directly used for crop improvement without purifying the peptide or proteins (Desai et al., 2010; Giddings et al., 2000). The heterologous production of radish Rs-AFP2 defensin in tobacco protected the transgenic tobacco from Alternaria longipes (Terras et al., 1995), whereas the alfalfa anti-fungal peptide (alfAPF) defensin reduced the infection in transgenic potato plants against the fungal pathogen Verticillium dahliae (Gao et al., 2000).

Due to their antimicrobial activity and complex structures, several AMPs cannot be produced in microbial systems. For example, retrocyclin-101 (RC101) and Protegrin-1 (PG1) can be used as therapeutic agents against bacterial and/or viral infections; none has yet been produced in microbial systems, but both have been expressed in the tobacco chloroplast with a yield of around 20% of total soluble protein and retention of antiviral activity (Lee et al., 2011b). The largescale production of AMPs can be applied in numerous industries such as biotechnology, pharmaceuticals, cosmetics and food industry. However, the limiting factor is the high production costs of some AMPs due to their molecular mass and protein folding characteristics.

The AMP can be degraded by endopeptidases, which also limits their bioactivity. Thus, selecting the right system is essential for large-scale production and functional recombinant AMPs. Plant-based expression systems are promising platforms for further engineering and production of AMPs (Parachin *et al.*, 2012). All AMPs heterologously produced in *N. tabacum* are listed in Table 3.

CONCLUSION

In conclusion, plants can act as 'biofactories' for the production of recombinant proteins, to reduce the production costs due to the low maintenance cost and high quality of the therapeutic proteins (Davies, 2010; Gomord and Faye, 2004; Gleba, 2007; Ling *et al.*, 2010; Mett *et al.*, 2008; Melnik and Stoger, 2013; Niknejad

et al., 2016). Moreover, several remarkable advances make plant expression system an attractive high-tech platform for plant-made protein pharmaceuticals (PMPs).

ACKNOWLEDGMENTS

The author acknowledges Prof. Mrinal Bhave and Dr. Daine Webster, Swinburne University of Technology, Melbourne, Australia.

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How to cite this article:

Niknejad, A. 2018. Plant-based expression systems for protein and antimicrobial peptide production. – Nova Biol. Reperta 5(3): 262-273.