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Plant molecular pharming is a promising system for cost-effective production of veterinary vaccines

Bitki moleküler üretimli ilaçlar, veteriner aşılarının uygun maliyetli üretimi için umut verici bir sistemdir

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ABSTRACT

Vaccination of animals has been used for centuries and is generally considered the most cost-effective and sustainable method of disease control and prevention. About twenty-five years ago, vaccines were in a inactive form or live attenuated organisms and often were not very effective. Advances in molecular biology and biotechnology have made it possible to develop new vaccines and therapeutic targes. Plant expression system has been demonstrated to be a promising platform for production of a variety of recombinant proteins such as vaccines, antibodies, therapeutic proteins, human and industrial enzymes, toxins etc. for health, agricultural and industrial applications. Although plant produced products are already available and licensed for human use, however, there are currently no plant-based vaccines on the market for animal use other than the Newcastle poultry vaccine. This is probably explained by relatively high cost of plant produced recombinant protein based vaccines for animal use. Therefore, the development of inexpensive and affordable plant-based vaccines and their formulation is very important for the production of economical animal vaccines. In this review, (1) different expression systems, (2) the history of plant-based expression systems, (3) different types of vaccines, and(4) plant-based animal vaccine production in plants are discussed. We also discussed the advantages of plants in the development of veterinary vaccines and new developed strategies that can lead to the production of cost-effective, stable and highly immunogenic veterinary vaccines.

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ÖZ

Hayvanların aşılanması yüzyıllardır kullanılmaktadır ve genellikle hastalık kontrolü ve önlenmesi için en uygun maliyetli ve sürdürülebilir yöntem olarak kabul edilmektedir. Yaklaşık yirmi beş yıl önce, aşılar inaktif bir formdaydı veya canlı zayıflatılmış organizmalardı ve çoğu zaman çok etkili değildi. Moleküler biyoloji ve biyoteknolojideki gelişmeler, yeni aşılar ve terapötik hedefler geliştirmeyi mümkün kılmıştır. Bitki ekspresyon sisteminin, aşılar, antikorlar, terapötik proteinler, insan ve endüstriyel enzimler, toksinler vb. gibi çeşitli rekombinant proteinlerin üretimi sağlık, tarım ve endüstriyel uygulamalar için umut verici bir platform olduğu gösterilmiştir. Bitki tarafından üretilen ürünler zaten mevcut ve insan kullanımı için lisanslanmış olsa da, şu anda piyasada Newcastle kümes hayvanları aşısından başka hayvan kullanımı için bitki bazlı aşılar bulunmamaktadır. Bu muhtemelen hayvan kullanımı için bitki tarafından üretilen rekombinant protein bazlı aşıların nispeten yüksek maliyeti ile açıklanmaktadır. Bu nedenle, ucuz ve uygun fiyatlı bitki bazlı aşıların geliştirilmesi ve bunların formülasyonu, ekonomik hayvan aşılarının üretimi için çok önemlidir. Bu derlemede, (1) farklı ekspresyon sistemleri, (2) bitki bazlı ekspresyon sistemlerinin tarihçesi, (3) farklı aşı türleri ve (4) bitkilerde bitki bazlı hayvan aşısı üretimi tartışılmıştır. Ayrıca, bitkilerin veteriner aşılarının geliştirilmesindeki avantajlarını ve uygun maliyetli, istikrarlı ve yüksek immünojenik veteriner aşılarının üretimine yol açabilecek yeni geliştirilmiş stratejileri tartışılmıştır.

1. Introduction

The basis of the vaccination concept is to mimic natural immunity. It is aimed to achieve a response similar to natural immunity by administering the agents that have lost their disease-causing properties to the body, but will provide immune enhancement. The term vaccine is derived from the Latin term vacca-cow. It was invented on the idea that the cow pox virus, first proposed by Edward Jenner, would protect people against the human smallpox virus (Meeusen et al. 2007). Until recently, vaccines were developed using conventional technologies. However, the introduction of modern biotechnology and genomics has enabled not only to know which antigen triggers immunity, but also to ensure host defense and the development of safer and more effective vaccines. In animal husbandry, vaccines have been an effective and safe method, especially in fight against viral diseases that cannot be treated with antibiotics, and especially in protection from diseases such as rabies transmitted from wildlife. (Rogan and Babiuk 2005). Mass vaccination of animals is particularly important for the prevention of zoonosis cases. Moreover, it reduces the use of animal drugs, reduces the side effects associated with drug use, minimizes environmental damage and prevents drug residues in animal products. In short, vaccination affects not only animal health but also human society health and its economic effects are quite high (Shams et al. 2005).The use of inactive or live attenuated organisms in traditional vaccination could not provide a safe and effective immunity. The development of DNA-based technologies created new opportunity for the development of protein expression systems. To date, several protein expression systems have been developed, and various recombinant proteins have been produced using these expression systems, including infectious disease vaccines, which are already on the market. The first expression system developed is the bacterial expression system and is currently the most used one. Although the bacterial expression system is cheap and efficient, the system has serious limitations for the production of eukaryotic proteins, especially complex mammalian proteins. Due to its low cost and safety, yeast is an excellent eukaryotic host for the production of recombinant proteins. However, hypermannosylation, which is common in yeast, adversely affects protein folding. Mammalian cell culture is ideal platform for production of complex mammalia proteins, however this system has a risk with mammalian pathogen contamination, is very expensive, and also difficult to scale-up. Because of a number of disadvantages, other expression systems including plants that have a high production capacity and provide production of highly soluble, correctly folded proteins with eukaryotic type of post translational modifications, are continually being developed. So far a number of recombinant proteins, including vaccine antigens, antibodies, and therapeutic proteins have been produced in plants. Plant expression system has many advantages compared to other expression systems, including simple, highly scalable, cost-effective manufacturing, relative product safety due to the lack of any harbored mammalian pathogens, and the presence of the eukaryotic post-translational modification (PTM) machinery and have the ability to accumulate grams of target protein per kilogram of biomass in about a week. Development of plant expression systems allowed to shift the production of some pharmaceuticals from bacterial, yeast expression systems and mammalian cell cultures to plants (Lico et al. 2012; Merlin et al.2014; Twyman et al. 2005). Some enzymes that are produced in plants have been already commercialized. For example, tobacco plant and maize have been used to manufacture human type I collagen (Shoseyov et al. 2014) and bovine trypsin (Sigma-Aldrich). Rice has been used to manufacture lactoferrin and human lysozyme (Hennegan et al. 2005; Yang et al. 2007b). In 2012, Protalix and its partner Pfizer received FDA approval to manufacture plant-derived taliglucerase alpha, which is used as an enzyme replacement therapeutic in persons who is suffering from Gaucher disease.

2. Types of vaccines

There are different types of vaccines that are used to prevent various diseases.

2.1. Live attenuated vaccines

In live attenuated vaccines the virus or bacteria has been weakened but is functional or alive, and therefore can be replicated in the body and generate an immune response without causing the disease, e.g. measles, chickenpox, rotavirus, mumps, rubella and shingles vaccine viruses. Live attenuated vaccines are developed in a cell that is not a natural host and after certain processes in various environments, are transferred to their natural host and it is hoped that they will generate an adequate immune response. (Meeusen et al. 2007). However, live attenuated vaccines that fail to provide high protection also have many disadvantages such as re-virulence or inflammation. In addition, need of refrigerated storage gives an extra burden for distribution (Babiuk et al. 2003). It is difficult to achieve standardization as the production of virus vaccines requires the use of live cells, and their formulations are challenging as they can be in an enveloped or non-enveloped form. However, it is easier to manufacture than inactivated virus vaccines and there is no use of adjuvants (Gelder and Makoschey 2012).

2.2. Inactivated or dead vaccines

Inactivated or dead vaccines are obtained by formulating one or more dead bacterial agents or strains or dead virus in an oil or aluminum hydroxide adjuvant. It is more stable in field conditions and more economical to manufacture than live attenuated vaccines. Inactivation is achieved by denaturation of proteins or degradation of nucleic acids by various physical or chemical processes. It is then purified and mixed with an adjuvant. Inactivated vaccines do not contain live viruses or bacteria and safer than live attenuated vaccines, but since the replication of the pathogen is destroyed, it cannot provide a long-term protection (Cho et al. 2002; Gelder and Makoschey 2012). Inactivated vaccines usually require multiple doses.

2.3. RNA vaccines

RNA is a new type of vaccine that genetic elements (RNA) of a pathogen is inserted into human cells to produce pathogen antigens in order to elicit an adaptive immune response against a specific pathogen. The advantages of this vaccine are rapid production and increased cellular immunity (Kramps et al. 2020). However, there are several disadvantages associated with cold chain distribution and storage at low temperatures (for example, BioNtech/Pfizer COVID-19 vaccine has to be kept at minus 70 degrees Celsius). Moreover, RNA replication in the cells cannot be controlled. Notable, until now, no mRNA vaccine has been approved for use in humans.

2.4. Modern-protein based vaccines

The development of DNA based technologies and protein expression systems created new opportunity to design, develop and produce a new generation, protein-based vaccines. These vaccines are safe, can provide high immunogenicity and the dose of the vaccine can be controlled. However, it is challenging to produce functionally active antigens and associated with incorrect folding of some complex proteins (especially proteins with many disulfide bridges) produced in different host expression system. Therefore, for production of functionally active protein antigens more flexible approaches are required (Mamedov et al. 2012; Mamedov et al. 2017; Mamedov et al. 2019a).

3. Plant expression system: advantages, limitations and solutions

Recombinant proteins in plants were first produced by introduction of a target gene of interest into the nuclear genome (Franken et al. 1997; Daniell et al. 2001) and in other words by stable transformation. In a stable transformation, gene of interest is integrated into plant genome. Notable, stable transformation has number of disadvantages including the long development time, and low level accumulation of target proteins. In addition, stable transformation has an environmental concern associated with contamination of wild types; the possibility of gene flow from transgenic plants to wild types. Due to these disadvantages, another type of plant expression system, transient gene expression system has been developed (Yusibov and Mamedov 2010). The transient plant expression platform has several advantages over stable transformation. The transient plant expression system is fast, has high performance, and provides uniformity and consistency of target accumulation, has scalability, and has less environmental concerns. Although plant transient expression system is promising expression system, however this system had serious limitations for the production of a wide range of bacterial antigens, malaria antigens, some human proteins etc. that do not require N-glycosylation. The problem has recently been addressed through the development of an economical and practical technology that provided production of proteins of interest in native-like, non-glycosylated forms. This was achieved by *in vivo* deglycosylation of target proteins of interest by co-expression with bacterial PNGase F (Mamedov et al. 2012; Mamedov and Yusibov 2013; Mamedov et al. 2016) or with Endo H enzymes (Mamedov et al. 2017; Mamedov et al. 2019b).Using this technology, a functional active Pfs48/45 antigen of *Plasmodium falciparum* has been produced in plants and mice immunized with this antigen showed strong inhibition in standard membrane-feeding assay (SMFA) analysis for the first time (Mamedov et al. 2019a and b). This technology has been also applied for production of protective antigen of *Bacillus anthracis* (Mamedov et al. 2012; Mamedov et al. 2016; Mamedov et al. 2017; Mamedov et al. 2019a). Notably, PA contains six potential N-linked glycosylation sites, however PA is not glycosylated in the native host. When expressed in any eukaryotic system including plants, PA is aberrantly glycosylated (Mamedov et al. 2016). In earlier studies on PA, it was demonstrated that plant produced glycosylated PA83 protein was not functionally active and could not form lethal toxin (LeTx) *in vitro* (Chichester et al. 2013). However, deglycosylated PA83, produced by PNGase F co-expression was functional active could form lethal toxin (LeTx) *in vitro* (Mamedov et al. 2016). In addition, deglycosylated forms of PA83 was much more stable (Mamedov et al. 2016; 2017) and immunogenic (Mamedov et al. 2016) compared to glycosylated counterpart. Thus, the *in vivo* enzymatic deglycosylation strategy offers new opportunities for the production of economical, stable and highly immunogenic veterinary vaccines in plants. It should be noted that, since chloroplast proteins are not glycosylated, therefore, chloroplasts can be also used for production of recombinant proteins including vaccine candidates in non-N-glycosylated form (Tregoning et al. 2004; Koya et al. 2005; Molina et al. 2005; Daniell 2006). However, this system has several disadvantages associated mainly with low level expression of target proteins.

4. Veterinary protein based vaccines produced in plants

Enterotoxin (LT-B) produced in transgenic tobacco or potatoes (Haq et al. 1995), were the first veterinary antigens proven that these antigens can be produced in plants. Production of these antigens in plants provided also the first proof of principle for edible vaccines. Later, mink enteritis virus (MEV) VP2 capsid protein/antigen was produced in bean plants (Dalsgaard et al. 1997). Castanon et al. (1999) reported about expression of rabbit hemorrhagic disease virus (RHDV) VP60 capsid protein in transgenic potatoes. Foot and mouth disease virus (FMDV) caused by an Aphthovirus of the family Picornaviridae is a severe, highly contagious viral disease that causes foot and mouth disease (Carrillo et al. 2005) and infects mammals such as cattle, pig, sheep, goat, and other cloven-hoofed animals.VP1-derived peptide of FMDV was recombinantly produced in various hosts including plants (Li et al. 2006). Thus, engineering of VP1 protein would be important to develop cost effective, safe and highly immunogenic vaccine against foot and mouth disease using transient expression technology. A rotavirus vaccine have been produced in transgenic potato as a fusion protein (cholera toxin B and A2 subunits fused with murine rotavirus enterotoxin and enterotoxigenic *Escherichia coli* fimbrial antigen (Yu et al. 2001) and it was demonstrated after oral immunization with this fusion protein in mice elicited serum and intestinal antibodies. In another study, bovine rotavirus (BRV) VP4 as His tagged protein was produced in *N. benthamiana* (Filgueira et al. 2004). A fusion protein consisting of a short peptide derived from BRVVP4 fused to GUS was expressed in transgenic alfalfa (Wigdorovitz et al. 2004). It was shown that when this antigen given intraperitoneally and orally to adult female mice, their sucklings were protected against challenge (Wigdorovitz et al. 2004). Human rotavirus VP6, produced in transgenic alfalfa, also protected mice from simian rotavirus infection. The VLP-based vaccine, the main capsid protein L1 of the human papillomavirus, has been produced in transgenic tobacco or potato (Biemelt et al. 2003; Varsani et al. 2003; Warzecha et al. 2003) protected against challenge.

There were attempts to produce functional active recombinant protein based vaccine against Rabies virus in plants (McGarvey et al. 1995; Loza-Rubio et al. 2008; Yusibov et al. 2002). Notable, rabies virus consists a single-stranded, negative-sense RNA genome, which encodes five structural proteins designated N (nucleoprotein), P (phosphoprotein), M (matrix protein), G (glycoprotein), and L (RNA-dependent RNA polymerase) (Schnell et al. 2010). It has been confirmed that glycoprotein G is responsible mainly for the induction of protective immunity and represents the major antigen of RABV (Wiktor et al. 1973; Cox et al. 1977; Macfarlan et al. 1986;

Foley et al. 2000). Glycoprotein G of RABV assembles in the form of homotrimers on the surface of RABV or of the infected cells and is the target for binding virus-neutralizing antibodies, thus harbor the major antigenic determinants of the virus (Gaudin et al. 1992). For this reason, most of the recombinant candidate vaccines studied so far are based on RABV-G protein. RABV-G protein have been produced with different expression systems (Dietzschold et al. 2003; Ashraf et al. 2005; Kaur et al. 2010; Huang et al. 2011). It was reported that even a single immunization of G protein was sufficient to induce high RABV-specific virus-neutralizing antibody titers in dogs, cats and mice. Therefore, G protein is a leading candidate for a new generation, protein based, subunit vaccine. Currently, although human and animal vaccines against RABV are available, allowing effective rabies control. However, they are very expensive and have relatively poor immunogenicity. In addition, protection is conferred after multiple immunizations with high antigen doses. Therefore, more immunogenic, safer and cheaper rabies vaccines are urgently needed. At this point, plant expression system would be also ideal for production of low cost, safe, stable and highly immunogenic vaccine against rabies. An artificial polypeptide containing rabies virus G protein (aa 253-275), and N protein (aa 404-418), fused with Alfalfa mosaic virus (AMV) CP were expressed either in *Nicotiana tabacum* plants transgenic for AMV replicase, or viar TMV in either *N. benthamiana* or spinach (Yusibov et al. 2002). It should be noted that the full length G protein of ERA rabies was previously produced in transgenic plants (McGarvey et al.1995; Loza-Rubio et al. 2008), however, the level of antigenic protein was low. Thus, engineering and developing full length G protein based plant produced vaccine would be important to produce cost effective, safe and highly immunogenic vaccine against rabies.

There have been many efforts to develop recombinant protective antigen based vaccine against anthrax. In 2002 protective antigen (PA) of *Bacillus anthraces* was first expressed in transgenic *N. tabacum* (Aziz et al. 2002). Later, PA was produced in transplastomic *N. tabacum* that significant increased the yield of expression (Aziz et al. 2005).The expression level of PA expressed in chloroplast by Henry Daniell's group were high, ~2.5 g kg^{-1} in fresh leaf tissue. Chloroplast-derived PA with adjuvant produced high IgG titers and survived challenge with lethal doses of toxin (Koya et al. 2005). Later, PA was produced in *N. benthamiana* plant using transient expression system as a N- glycosylated protein. It was shown that glycosylated form of PA83, expressed in plants could not form lethal toxin (LeTx) *in vitro* (Chichester et al. 2013). However, PA83 produced by *in vivo* deglycosylation technology (co-expression with bacterial PNGase F), (Mamedov et al. 2012) was functional active and was more immunogenic compared to glycosylated form of PA (Mamedov et al. 2016). It was demonstrated that deglycosylated forms of PA83, produced by both PNGase F (Mamedov et al. 2012) or Endo H (Mamedov et al. 2017) in *N. benthamiana* plant was much more stable (Mamedov et al. 2016) compared to glycosylated form of the same protein (Chester et al. 2015).Thus, PNGase F or Endo H deglycosylated forms of PA are promising candidates for the development of a cost-effective, safe and immunogenic anthrax vaccine for use in livestock. It should be noted that the Sterne (34F2) *Bacillus anthracis* strain was developed in the 1930s and since then this vaccine has been used as a predominant method of immunizing livestock against anthrax worldwide. It is administered to livestock in a dose containing up to 10 million viable spores. Can the Sterne strain cause infections in people? Theoretically, yes. The reason why this vaccine is still used in livestock today is that an economical and safe anthrax vaccine for use in livestock has not been developed. At this point, the plant expression system could be a promising platform for the economical production of a protein-based vaccine against anthrax for use in livestock.

5. Conclusion

Plant expression system could be promising platform for the production of cost effective, safe, stable and highly immunogenic veterinary vaccines. A number of veterinary vaccines have been produced in plants. However, the development of strategies for the expression of inexpensive and affordable plant-based vaccines and their formulation is very important for the production of economical veterinary vaccines. Plant expressions system had serious limitation for production of those proteins that do not require N-glycosylation in the native host, including a wide range bacterial proteins, enzymes, toxins etc. The limitation was solved by developing a robust strategy for production of proteins in plants in non-N-glycosylated form by co-expressing of target proteins of interest with bacterial deglycosylation enzymes PNGase F and Endo H. In fact, PA83 vaccine candidate, which was produced using Endo H enzymatic deglycosylation strategy is the most advanced vaccine candidate against anthrax in terms of cost, safety, stability and immunogenicity and is a promising vaccine candidate for human and veterinary use. Thus, the strategy of in vivo enzymatic deglycosylation in combination with flexible approaches can ensure the production of cost-effective, safe and highly immunogenic veterinary vaccines in plants.

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