Journal of Cell and Molecular Research (2015) 7 (1), 59-67

Research Article

Recombinant Vaccine Production in Green Plants: State of Art

Maziar Habibi-Pirkoohi[1], Afsaneh Mohkami[2*]

1. Department of Biotechnology and Plant Breeding, Ferdowsi University of Mashhad, Mashhad, Iran
2- Horticulture Research Institute, Shahid Bahonar University of Kerman, Kerman, Iran

Received 9 March 2015 Accepted 3 April 2015

Abstract

Green plants have emerged as ideal platforms for production of recombinant vaccine during recent decades. Various antigens relating to a large number of animal and human diseases have been studied in different plant species for production of recombinant vaccines. Despite the unique advantages of plant systems as green factories for production of recombinant vaccines, there are some major hurdles that have prevented commercial production of plant-based vaccines. In this review, theoretical background and practical applications of plant system for production of various recombinant vaccines are discussed.

Keywords: Recombinant Vaccine, Plant, Genetic Transformation

Introduction

Plant-derived pharmaceuticals (PDPs) are proteins or organic compounds produced in plants via recombinant DNA technology, which are used to improve human or animal health. Subunit vaccines represent one category of PDPs that have been validated in a variety of studies, including human clinical trials. Application of green plants for production of therapeutic products is an emerging field of biotechnology with high economic potential (Sala et al., 2003). Although vaccination with conventional vaccines proved to be an effective practice in prevention of diseases, yet there still is disagreement over its use. Some of the documented side effects of the elements and substances used in vaccine serums include: blood disorders, auto-immune diseases, cerebral palsy, brain damage, paralysis, neurological impairment, monkey fever, autism, mental retardation, premature aging, as well as others. Thus, there is an urgent need to find an alternative to the present vaccines. This alternative can be substituted by development of plant vaccines (Schillberg et al., 2005).Considering recent developments in genetic engineering and transformation methods, it is possible to develop a wide range of transgenic plants that can express various recombinant pharmaceutical compounds including viral and bacterial antigens, antibodies, and many other therapeutic proteins (Awale et al., 2012).

For a long time, Recombinant vaccines were exclusively produced in expensive expression platforms such as yeast or mammalian cells. High costs associated with preparation culture media and the risk of contamination by human pathogens are regarded as the major disadvantages of such systems. Production of recombinant vaccines in bacterial systems, though simple and cost-effective, was not successful due to improper folding of eukaryotic peptides and occurrence of inclusion bodies in bacterial hosts (Franklin and Mayfield, 2005). Genetic engineering of higher plants was a turning point in the field of recombinant vaccine production. The goal is to produce transgenic plants that upon oral or parenteral administration induce an immune response in the body. The first report of expressing a vaccine antigen within plants was published in 1990 when Curtiss and Cardineau expressed the Streptococcus mutants surface protein antigen A (SpaA) in tobacco (Curtiss and Cardineau, 1990). This pioneer study was followed by plant expression of the hepatitis B surface antigen (HbsAg) (kapusta et al., 1999), the *E. coli* heat–labile enterotoxin responsible for diarrhea (Haq et al., 1995), and the rabies virus glycoprotein (McGarvey et al., 1995). Proteins produced in these plants induced synthesis of antigen specific mucosal IgA and serum IgG when delivered orally to mice and humans.

Corresponding authors E-mail:
* amohkami@uk.ac.ir

Compared to other recombinant protein expression systems, Plants offer several advantages including the possession of eukaryotic posttranslational modification machinery, suitable folding of foreign protein, low cost scale up, target protein stability and safety of use of plant-derived products due to the lack of any mammalian pathogens. The cost of vaccine production in plant systems is comparable to that of microbial bioreactors and much lower than in mammalian cells. More importantly, in contrast to microorganisms, especially bacteria, it was well documented that plants express eukaryotic proteins in properly folded, modified, assembled and, consequently, native and biologically active forms. Plant-based recombinant vaccines are also advantageous in terms of safety, as naturally free of microbial toxins and human and animal pathogens (Pniewski et al., 2012). However, oral immunization is thought to be the largest benefit and, in the most enthusiastic plans, plant-based vaccines are to be used as edible vaccines(Awale et al., 2012).

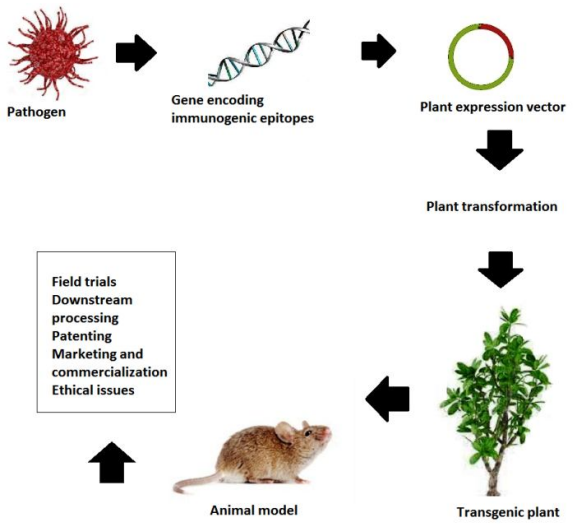

Figure1. Schematic representation of recombinant vaccine production in plat systems

Here, we first describe the principles of plant-based recombinant vaccine production. A handful examples demonstrating successful expression of antigen in plants are also cited. Moreover, strategies toward enhancing expression level will be noted, and finally, biosafety issues and future perspectives for commercial production of recombinant vaccines are discussed.

Plant species used for vaccine production

Plant-based vaccines are subunit vaccines in which the antigen of interest is expressed in plant tissues. The antigen, or antigens, must be expressed at a sufficiently high level in the chosen plant to allow for the practical oral delivery of a sufficient antigen dose to induce immune response. Many species can be adopted for production of recombinant vaccines, with tobacco being the most widely used host plant to date (Habibi and Zibaee, 2013). The advantages of the leafy crop tobacco include the high biomass yield, the ease of stable transformation either by cocultivation with *Agrobacterium tumefaciens* (Dugdale et al., 2014) or transiently by infiltration with transgenic agrobacteria (Leckie et al., 2011) or transfection with viral vectors (Rybicki et al., 2014). Another benefit is that tobacco is not used as a food crop, ensuring that a transformed line expressing a highly potent drug will not contaminate food resources. Other examples of leafy crops used for production of recombinant vaccines include alfalfa (Wigdorovitz et al., 1999), white clover (Lee et al., 2001), spinach (Yusibov et al., 2002; Karasev et al., 2005), lettuce (Kapusta et al., 2001), etc.

Tomato (*Solanumlycopersicum*) is an example of garden crops whose fruits are used for vaccine production. Tomato possesses a high fruit biomass yield and offers other advantages in terms of containment, because the plant is often grown in greenhouses. The most widespread use of tomato fruits in molecular farming has been in the expression of vaccine candidates. The first report on production of recombinant vaccines in tomato was the expression of rabies surface glycoprotein, which achieved the relatively high expression level (McGarvey et al., 1995). Other examples include cholera toxin B subunit (Jani et al., 2002) respiratory syncytial virus-F protein (Sandhu et al., 2000), toxin co-regulated pilus subunit A (TCPA) of *Vibrio cholera* (Sharma et al., 2008), as well as others. Other examples of fruits and vegetables used for antigen expression include potato (Mason et al., 1998; Yu et al., 2001), lettuce (Dong et al., 2014), carrot (Mendoza et al., 2011), etc.

Seed crops including both cereals (maize, rice, wheat and barley) and the grain legumes (soybean, pea, pigeon pea and peanut) have been used as ideal plant systems for production of recombinant vaccines (salaet al., 2003). The main advantage of seed crops is that recombinant proteins can be directed to accumulate specifically in the desiccated seed which is a natural storage organ, with the optimal biochemical environment for the accumulation of large amounts of protein. Moreover, recombinant proteins expressed in seeds have been shown to remain stable and active after storage at room temperature for over three years. Finally, seed proteome is fairly simple, which

reduces the likelihood that contaminating proteins will co-purify with the recombinant protein during downstream processing (Lamphear et al., 2004). According to Stoger et al (2000), Several factors should be considered when choosing an appropriate seed expression host, including geographical considerations, the ease of transformation and regeneration, the annual yield of seed per hectare, the yield of recombinant protein per kilogram of seed, the production cost of the crop, the percentage of the seed that is made up of protein and, inevitably, intellectual property issues.

Green microalgae have also emerged as new cell factories for production of recombinant vaccines. Microalgae possess advantages of prokaryote and eukaryote organisms simultaneously. On one side, they are unicellular organisms with very fast growth which facilitates mass production in short time (prokaryotic feature). On the other side, they are eukaryote and are able to process long eukaryotic peptides with accurate folding and appropriate post transcriptional modification (Specht et al., 2010). Examples of recombinant vaccine produced in microalgae include expression of Food and Mouth Disease Virus (FMDV) VP1 antigen in *Chlamydomonas reinhardtii* (Habibi et al., 2014), fusion protein containing the VP1 gene and the cholera toxin Bsubunit (Sun et al., 2003) and syndrome virus protein 28(VP28) (Surzycki et al., 2009) in chloroplast genome of the same species.

Several issues should be considered when selecting a plant species as an antigen expression host. The first issue is the form of vaccine delivery. Foreign proteins can be expressed in fresh tissue, such as mature plant leaves and germinating seedlings or in dry tissue, such as the seeds of cereals (Streatfield et al., 2001). Hydroponic culture is another ideal platform because the system makes it possible to secrete the expressed protein into the surrounding medium (Borisjuk et al., 1999). The plant species selected as expression system should possess optimum antigen expression, allows for cost-effective production, and can be manufactured into a practical form for oral delivery.

Plant transformation strategies

In general, recombinant subunit vaccines can be produced in plants either by stable or transient transformation. Stable transformation is the most common method widely practiced for production of transgenic lines expressing the antigen of interest. In this approach, the gene of interest is integrated in nuclear or plastid genome using biolistic or *Agrobacterium* mediated transformation methods. In *Agrobacterium* mediated gene transfer, the gene of interest is inserted into the T-region of a disarmed Ti plasmid of *Agrobacterium tumefaciens*. The recombinant DNA is placed into *Agrobacterium*; a plant pathogen which is co-cultured with the plant cells or tissues to be transformed. The main disadvantage of this method is that it gives low yield and the process is slow. This method works especially well for dicotyledon plants like potato, tomato and tobacco. In this manner, the foreign antigen is stably inherited through successive generations (Lal et al., 2007). Some agronomically important plant species (e. g. most cereal crops)are recalcitrant to *Agrobacterium* transformation, and a biolistic method is frequently used for these plants (Awaleet al., 2012). In this approach, DNA coated gold particles are propelled into plant cells using compressed helium gas and becomes incorporated into chromosomal DNA. The biolistic method usually results in higher-copy-number plants compared to those generated by *Agrobacterium*, which can enhance expression. However, excessive copy numbers or very high-level expression of nuclear genes can cause gene silencing, resulting in low protein accumulation. Thus it is important to select transgenic lines that carry only between one and three copies of the transgene (Sala et al., 2003).

In transient transformation technique, the epitope of interest is engineered into a plant virus, usually within the coat protein gene. Infection of target plant by this viral vector results in intracellular production and accumulation of the epitope. The epitope sequence, as well as the viral genome, never become integrated into the plant genome and hence are only expressed by the generation of infected cells (Yusibov et al., 2002). This approach has been successfully applied to tobacco, black-eyed beans and spinach (Dalsgaard et al., 1997).The potential advantage of viral expression systems compared to stable plant transformation is that viral replication can greatly amplify the template for protein synthesis resulting in high-level protein accumulation (Pniewski, 2014).Transient transformation can also be achieved by *A. tumefaciens*. This method, called “agroinfiltration”, involves the injection or vacuum infiltration of plants parts with a suspension of bacteria harboring the antigen of interest. This approach has a wide spectrum of applications and has been used for the study of molecular processes and production of interesting molecules of monoclonal antibodies (Orzaez et al., 2006), antigens of human (Mett et al., 2008) and livestock (Habibi et al., 2014) pathogens.

A newly developed transformation approach called

Magnifection is being used to overcome the limitations possessed by early platforms. It combines the two technologies namely agroinfiltration method and Tobacco Mosaic Virus (TMV)- based viral vectors system. This new approach allows the scalable production of a desired protein with high expression level and yield, low up- and downstream costs, reduced time, and most of all, reduced biosafety concerns (Gleba et al., 2005).

Enhancing antigen expression level in plants

Despite considerable advantages of green plants as feasible platforms for production of recombinant vaccine, low level of transgene expression is still a main drawback hindering commercial application of plant systems (Kang et al., 2003).As remarked by Habibi-Pirkoohi and Zibaei (2013), enhancing transgene expression in plant tissue will be a milestone in production of plant-based recombinant vaccines (Habibi-Pirkoohi and Zibaei, 2013).

To achieve this, several approaches have been proposed such as codon optimization, the use of strong plant promoters and untranslated leader sequences (Chikwamba et al., 2002).

Codon optimization is an efficient way to enhance transgene expression level in transgenic plants as different organisms prefer different codons when making a functional protein (Jabeen et al., 2010). It has been reported that codon optimization can enhance expression level in nuclear transformation as high as 5-fold (Fuhrmann et al., 1999) or up to 80-fold in chloroplast transformation (Franklinand Mayfield, 2005). Moreover, existence of rare codons in some organisms significantly reduces translation efficiency in transgenic plant (Gustafsson et al., 2004). Thus it is not surprising that many investigators make use of synthetic gene with optimized codon sequence (Habibi et al., 2014; Kang et al., 2004).

Presence of leader sequence at 5′ untranslated region is also efficacious in enhancing expression level. The prominent Kozak leader sequence (GCCACC) is a ribosome binding site (RBS) whose role in promotion of translation efficiency is well documented (De Angioletti et al., 2004). The upstream leader of Tobacco Mosaic Virus (TMV) called Ω sequence is another untranslated region which plays as a translational enhancer in higher plants. The CAA region residing within Ω sequence is responsible for translational enhancement and acts as a binding site for HSP101 heat shock protein, with the latter is necessary for translation improvement (Gallie, 2002).

In some cases, signal peptides such as SEKDEL sequence have been used to target the antigen in to endoplasmic reticulum (ER), where necessary enzymes and cellular machinery for proper folding are present(Xu et al., 2011). By addition of ER signals to transgene, high level of antigen expression has been observed in a number of studies (Kang et al., 2004; Haq et al., 1995; He et al., 2012). The ER signals are often attached to 3′ end of the transgene just before stop codon(Habibi et al., 2014).

Chloroplast transformation is an effective way to improve foreign antigen accumulation in plant tissues. This approach- usually referred to as cpDNA transformation- is based on the integration of the transgene into the circular chloroplast DNA (cpDNA) that is present in multiple copies in plant cells. Advantages of chloroplast engineering are numerous: the cpDNA molecule is completely sequenced in a number of important plants and is present to up to 10.000 copies per cell. Moreover, it has been shown that chloroplasts can properly process eukaryotic proteins, including correct folding and disulfide bridges (Daniel et al., 2001). Integration into cpDNA has two important advantages, the first being the foreign sequence is targeted to a precise cpDNA site by homologous recombination. This eliminates variability in gene expression and gene silencing, which often occurs in nuclear transformation. The second advantage is the enhanced accumulation of the recombinant antigen. Accumulation of recombinant protein in chloroplast engineering is far more than that of nuclear transformation (Ruhlman et al., 2010).

Oral delivery and mucosal immunity

The majority of infectious agents enter the body through mucosal membranes. Induction of mucosal immunity is best achieved by direct vaccine delivery to mucosal surfaces (Carter and Langridge, 2002). Orally delivered, non-replicating subunit vaccines have not yet achieved commercial success using any means of manufacture. The main hurdle facing the use of orally delivered immunogenic proteins is the likelihood that some proteins will be degraded after ingestion and that some immunogens may not be recognized efficiently at mucosal immune effect or sites in the gut. Although this is a potential limitation, the use of plants as a protein biomanufacturing system offers advantages in that the cost of obtaining the end product is comparatively low. Plant-derived vaccines have demonstrated the ability to induce both systemic and mucosal immune responses (Kong et al., 2001). The major obstacle to oral vaccination is the digestion of the antigenic protein in the stomach. Vaccines derived from plant cells have been shown to overcome this problem through the protective

effect of the plant cell wall. Like liposomes and microcapsules, the plant cell wall allows gradual release of the antigen onto the vast surface area of the lower digestive tract (Streatfield, 2006).

Diseases targeted for recombinant plant based vaccines

A large number of diseases have been studied for production of recombinant vaccines. This includes both human and animal infectious diseases and various plant species have been investigated as the host for production of recombinant vaccines. Foot and Mouth Disease (FMD) and hepatitis B are two example of disease for which many investigations have been carried out in trying to obtain an effective plant-based recombinant vaccine. A summary of plant-derived recombinant vaccines is presented in table 1. The table shows that *Agrobacterium*-mediated nuclear transformation is the dominant procedure for achieving transgenic plants and tobacco, potato and tomato are among the species widely used for recombinant vaccine production. The long list of recombinant vaccines is undoubtedly is not limited to the examples presented in this review, and the list is growing fast by introducing new vaccines, more sophisticated gene construct designs and application of new plant species.

Safety issues, Public acceptance and Commercialization

Plant-derived vaccines are free from human and animal pathogen contaminants. Furthermore plant DNA is not known to interact with the animal DNA and plant viral recombinants do not invade mammalian cells. Nevertheless, some concerns need to be addressed before recombinant vaccines release in market. One of the fears is that GM-pollen may escape to the nature and bear harmful influences on biodiversity. To address this concern, some pollen containment approaches have been developed which are often based on establishment of different forms of male sterility (Sala et al., 2003).

Analternative way of solving the problem is engineering vaccines in to the cpDNA, which is not transmitted to the sexual progeny through the pollen grains (Daniel et al., 2011). With this approach, land needed for industrial plant-derived vaccine-production will be in the order of a few thousand square meters because expression level of the antigen is of high magnitude. This enables vaccine-producing transgenic plants to be set apart from field grown crop plants. Another public concern in GM-plants is the presence of antibiotic resistance genes (used as selective marker in most transgenic plants). Approaches have now been developed to generate GM-plants (with both nuclear or cpDNA integration)that do not carry these genes (Puchta et al., 2000).

Table 1. Recombinant vaccines produced in transgenic plants

Antigen	Transformation method	Plant host	Reference
E. coli heat labile enterotoxin (LT-B)	*Agrobacterium*-mediated	Potato	(Mason et al., 1998)
E. coli heat labile enterotoxin (LT-B)	*Agrobacterium*-mediated	Tomato	(Walmsley et al., 2003)
E. coli heat labile enterotoxin (LT-B)	*Agrobacterium*-mediated	Soybean	(Moravec et al., 2007)
Foot and Mouth Disease antigen VP1	*Agrobacterium*-mediated	Alfalfa	(Wigdorovitz et al., 1999)
Foot and Mouth Disease antigen VP1	Chloroplast transformation (biolistic)	Tobacco	(Li et al., 2006)
Foot and Mouth Disease antigen VP1	Agroinfiltration	Tobacco	(Habibi et al., 2014)
Foot and Mouth Disease antigen VP1	*Agrobacterium*-mediated	Microalgae (*C. reinhardtii*)	(Habibi et al., 2014)
Hepatitis B antigen HBsAg	*Agrobacterium*-mediated	Potato	(Kong et al., 2001)
Hepatitis B antigen HBsAg	*Agrobacterium*-mediated	Lettuce	(Koprowski et al., 2001)
Hepatitis B antigen HBsAg	*Agrobacterium*-mediated	Lupin (*Lupinuslute us*L.)	(Kapusta et al., 1999)
HIV glycoprotein	*Agrobacterium*-mediated	Spinach	(Karasev et al., 2005)
HIV antigen p24	*Agrobacterium*-mediated	Potato	(Obregon et al., 2006)
Rabies G and N proteins	*Agrobacterium*-mediated	Tobacco	(Yusibov et al., 2002)
Rinderpest virus hemagglutinin (H)	*Agrobacterium*-mediated	Tobacco	(Khandelwal et al., 2003)
Newcastle disease F antigen	biolistic	Maize	(Guerrero-Andre et al., 2006)
Respiratory Syncytial Virus (RSV) antigens F and G	*Agrobacterium*-mediated	Tomato	(Sandhu et al., 2000)

Despite numerous advantages of plant-based recombinant vaccines, none of the major pharmaceutical companies is directing funding towards the development of plant-derived vaccines for infectious diseases. This reluctance about commercial production of plant-based recombinant vaccines is mainly due to concern about the potential for significant return on investment; uncertainties in the regulatory processes; limited human clinical trial data that establish required dosages, timing of delivery, and evaluation of possible adverse immunological effects; and finally, a lack of personnel with sufficient expertise in plant biology (Zhang et al., 2011).Participation of both the public sector and the non-profit sector will be essential to provide leadership and investment support to unlock the potential of plant-derived vaccines.

Conclusion

Application of green plants for production of recombinant vaccines offers many advantages over traditional methods making this approach a practical way for manufacture of mucosal vaccines on a global scale. Since the pioneer work of Curtiss and Cardineau (1990), many vaccine antigens have been expressed in different plant species to demonstrate the feasibility of oral plant-based vaccines. Despite the promising future and several successes achieved in this field, different issues will have to be established and well defined such as high expression levels, product quality, downstream process costs, regulatory framework, efficacy and safety. Moreover, a large part of the researches in the field of recombinant vaccine production are carried out in tobacco which is not an edible plant and, due to possessing high level of alkaloids, is not affordable as an oral vaccine. Thus, it is necessary to try other plants such as fruits and vegetables to realize production of a plat-based recombinant vaccine.

Growing progress in the field of biotechnology and plant genetic engineering will undoubtedly assist in improvement of plant-based recombinant vaccines.

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