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The methods behind transgenic plant production: a review

Jasmin Šutković[1], Nour Hamad[2], Petar Glamočlija[3]
[1,2] International University of Sarajevo, Genetics and Bioengineering,
[3] University of Sarajevo, Faculty of Agriculture and Food Sciences

ABSTRACT

The ability to insert a gene into a plant's nuclear or chloroplast genome enables the transformation of higher plants (e.g. tobacco, *Arabidopsis thaliana*, potato, tomato, and banana) into Bioreactors for the production of plant-derived pharmaceuticals. Biopharmaceuticals are generally produced on a commercial basis by scale fermentation in bacteria, yeast, or animal cells. Several plant-derived pharmaceuticals have undergone clinical trials and are close to market authorization, with antibodies and vaccines being the front runners. Plant-derived vaccines have been produced using recombinant plant viruses as transgenic expression vectors and *Agrobacterium tumefacien*s transformation systems. During the last decade, several efficient plant-based expression systems have been examined, and more than 100 recombinant proteins, including plant-derived vaccine antigens. Besides, regulatory protocols are slowing down production. Industry requirements and public acceptance of the technology are important aspects in establishing successful products. This paper reviews the current status of development in the area of biopharmaceuticals and vaccines produced from transgenic plants.

Keywords: Transgenetics, Plant-derived vaccines, Microinjection, Biopharming, Agrobacterium

Corresponding Author:

Jasmin Šutković
Genetics and Bioengineering
International University of Sarajevo, Bosnia
E-mail: jsutkovic@ius.edu.ba

1. Introduction

Genetic transformation is the most relevant tool used for the analysis of plant genome. Through gene discovery development, the investigation of genetically controlled mechanisms and gene function is now a very common procedure. Furthermore, genetic transformation enables the introduction of foreign genes into crop plants, expeditiously creating new genetically modified organisms [1]. Infectious diseases account for more than 45% of total deaths in developing countries [2] and vaccination is the most effective means to prevent infectious diseases. It is estimated that more than 30 million children in the world are not immunized against treatable or preventable diseases because the currently used approaches to vaccine production are technologically complex and expensive. The possible solution is that vaccines will be based on plant expression systems and used for the production of pharmaceutical proteins. Humans have relied on plants as a source of medicines since antiquity, and up to 25% of drugs on the market today contain active pharmaceutical ingredients derived from plants [3]. Thus, the production of biologically active proteins and biopharmaceuticals is termed as plant molecular farming (PMF). In the recent decade, PMF became an attractive manufacturing system which can achieve relevant production in a short period [4, 5]. It is known that plant-produced pharmaceuticals are much less likely to contain human pathogens, and the cost of production is relatively low and easily scalable, where the products are efficiently harvested and stored [6]. Harvesting of pharmaceuticals from plants may be greatly facilitated by directing the expression of therapeutic proteins to the specialized storage organs of plants [7]. In recent years, several proteins have been

successfully produced in plants, which include human serum albumin [8], hemoglobin, monoclonal antibodies [9], viral or bacterial antigens (vaccine), encephalin, and trichosanthin. Plants are inexpensive compared to fermenter systems, more scalable, and in terms of safety they do not produce endotoxins like bacteria, nor do they support the proliferation of human viruses and prions like mammalian cells [10, 11]. The production of recombinant proteins in plants has improved in the past decade and the potential drawbacks with PMF have now been achieved [12-14].
This paper aims to state the current techniques used for plant gene delivery, emphasizing the pros and cons of plant vaccine production and delivery.

2. Gene delivery methods for plant transformation

Gene Transfer is the introduction of foreign genetic material, either DNA or RNA, artificially or naturally into a cell. It is often also referred to as transformation and one of the foundations of molecular biology. Plant transformation was first described in tobacco in 1984 [15]. Tobacco is said to be an ideal model system for the production of the therapeutic proteins and is considered as a biology workhouse of the plant word [16]. Following this, several techniques have been developed for delivering the transgene into the plants, including; vectors and viruses. To achieve genetic transformation in plants, we need the construction of a vector (genetic vehicle) which transports the genes of interest, flanked by the necessary controlling sequences i.e. promoter, terminator, a selectable marker, and other genes that deliver the DNA into the host plant (example; virgenes of *Agrobacterium*). The first marker used in this way was a gene encoding neomycin phosphotransferase (NPTII) which, when fused to a promoter allowing expression in plant cells, conferred resistance to kanamycin [17].

Gene transformation is achieved in several ways; one method DNA uptake is incubated into an isolated protoplast, controlled by chemical procedures, electroporation, or the use of high-velocity particles (particle bombardment). Those Direct DNA uptake techniques are useful for stable transformation as well as transient gene expression. However, the frequency of stable transformation is low when compared to transient transformation in which it takes a long time to regenerate whole transgenic plants [18].

Generally, we use the following gene delivery methods;

- Direct gene transfer method generation of the transgenic plants by stable integration of a transgene (nuclear or plasmid) in the plant genome [19].
- Indirect gene transfer by using plant viruses and plasmid such as vectors, and bacteria as vehicles, e.g. *Agrobacterium tumefaciens* [18].

2.1. Direct gene transfer methods

Direct DNA uptake is useful for both stable transformation and transient gene expression. However, the frequency of stable transformation is low, and it takes a long time to regenerate whole transgenic plants. Direct DNA uptake can be achieved by physical and chemical transfer as with DNA imbibition by cell, tissue, and organs. One of the physical techniques for gene delivery is *Electroporation.* This method generally utilizes protoplast because thick plant cell walls restrict macromolecular movements [20]. For transformation, both plasmid DNA and *Agrobacterium inoculums* can be applied. The first attempts to adopt methods employed in protoplasts for organized plant tissues were reported in the early nineties, and the main idea was to check the transient expression of a transgene under different organs or tissue-specific promoters. Efficient protocols for the electroporation of cell suspensions have been worked out for many species, e.g. tobacco, rice, and wheat. So far, the best results have been obtained for maize. Deshayes et al. (1985) transformed immature embryos and embryogenic callus type I, which were briefly digested in a solution of pectolytic enzymes, followed by transfer into electroporation cuvettes [21].

One of the most efficient direct gene transfer methods is *Gene gun particle bombardments* which is a vector-independent method. The gene gun method or a biolistic gene delivery system that includes particle bombardment is a commonly used method for genetic transformation of plants and other organisms. The main purpose and advantage of this method are enabling the DNA to enter cells, tissues, and intracellular

organelles which are naturally impermeable to foreign DNA, especially in plant cells [22]. The first Gene gun was produced at Cornell University in 1987 by Klein and Stanford. The gene gun is part of the gene transfer method called the biolistic (also known as particle bombardment) method. In this method the DNA or RNA is connected to biological inert particles (such as gold or tungsten), put on the target tissue in a vacuum condition, and accelerated by a powerful shot to the tissue, so effectively introduced into the target cells [23]. Importantly, the biolistic method can be achieved by two ways for antigen expression in plants, including; nuclear transformation and chloroplast transformation. Nuclear transformation is integrating the desired gene to the nucleus via non-homologous recombination, while chloroplast transformation via homologous recombination [24]. The advantage of this method is the stable integration of foreign DNA into the plant system. However, it might cause several damages to the plant tissue [24].

Further, it is possible to transfer the genes through the *Microinjection* method. This method is based on introducing DNA into the nucleus or cytoplasm with glass micro capillary-injection pipette. This operation requires a micromanipulator. Currently, microinjection is widely used for the transformation of large animal cells e.g. frog egg cells or the cells of mammalian embryos, whereas it has not been developed into a routine transformation method for plants. The procedure is very slow and requires an expensive micromanipulator. However, one of the unquestionable improvements of microinjection was allowing the introduction of DNA plasmids as well as the whole chromosomes into tobacco plant cells [25].

Besides, for direct methods in gene delivery, scientists have used Silicon carbide-fibre mediated transformation (SCMT), pollen-tube pathway (PTP) technology, and Lipofection method. SCMT is one of the least complicated methods of plant transformation. Silicon carbide-fibres are simply added to a suspension containing plant tissue (cell clusters, immature embryos, and callus) and plasmid DNA, and then mixed in a vortex. DNA-coated fibres penetrate the cell wall in the presence of small holes created in collisions between the plant cells, and fibres. The main disadvantages of this method are low transformation efficiency, damage to cells negatively influencing their further regeneration capability [26].The transformation method via the pollen-tube pathway (PTP) was firstly used for the transformation of rice. The authors obtained transgenic plants at remarkably high frequency. After plant pollination, the plant styles were cut out, and the DNA was added by gene gun so the DNA could reach the ovule by flowing down the pollen-tube [27].

Further, for gene transformation *Liposome* molecules are used, through the *Lipofection method*. Liposomes are circular lipid molecules with an aqueous interior that can carry nucleic acids. Liposomes encapsulate the DNA molecules and fuse to the cell membranes by direct adhering so the liposomes could transfer the DNA fragments. Therefore, the DNA can enter the interior of the cell and then move to the nucleus. Liposome mediated transformation is not simple, despite the low expense and equipment requirement. The reason lies in the fact that it requires a lot of labor work and has low efficiency. Only several reports on the integration of genes introduced using liposomes followed by transgenic plant regeneration for tobacco and wheat have been published thus far [28].
Direct gene delivery may also be achieved by Chemical transfer of DNA. Protoplasts are mainly used which are incubated with DNA in buffers containing chemical compounds such as polybrene–spermidine [29], Poly-Ethilen Glycerol (PEG) [27], Calcium-Phosphate [30], Diethyl amino ethyl (DEAE) [31] and polyethylene glycol [32].

Chloroplasts are one of the important targets for genetic engineering in which a study in 1995 engineered the tobacco chloroplast genome for herbicide-resistant [33]. Compared to this and above-mentioned methods, in a study conducted in 2019, the nanoparticle-mediated chloroplast transgene delivery into several plants without external biolistic or chemical aid in which chitosan-complexed single-walled carbon nanotubes was used [34].

2.2. Indirect gene transfer methods

The most widely used method for the introduction of new genes into plants is based on the natural DNA transfer capacity of *Agrobacterium tumefaciens* [35]. It was firstly isolated from a gall tissue which caused the crown gall disease and could infect more than 600 plants [36]. However, the first transgenic plant by *A. tumerfacient* was performed in 1980 [37]. Later on, more techniques of *A.tumefaciens*-mediated genetic transformation in crop were used to boost the agricultural biotechnology. The *Agrobacterium*-mediated gene transfer technique was used to introduce new traits to various plants through the gene transfer mechanism [38]. This bacterium can introduce part of its plasmid DNA (called transfer DNA or T-DNA) into the nuclear

genome of infected plant cells [35, 39]. Genes of interest are first introduced into *Agrobacterium tumefaciens* and later by infecting the plant cells the gene of interest is transferred to the plant genome. During this infection, a part of the Ti-plasmid of Agrobacterium, called T-DNA, is transferred and integrated into the plant genome. This natural capacity made us use this bacterium as a natural vector of foreign genes (inserted into the Ti-plasmid) into plant chromosomes [40]. Agrobacterium transformation is also possible with using the freeze/thaw method [41], a very simple and does not require special equipment, used together with electroporation [22]. Besides, Sonication methods can be used to form a wound on the plant to allow penetration of the *Agrobacterium* to plant tissue which could increase the plant cell infection [42].

3. Biopharming

Genetic engineering technology applied to plants was firstly used in 1988, for the production of plants capable of functioning as a bioreactor for protein production [3]. Technologies used for genetic manipulations of plant genomes have developed and diversified, including a variety of production platforms for specific target proteins, mostly biopharmaceuticals [43]. The process of pharmaceutical productions in plants is called biopharming. There are several gene transfer methods in biopharming, including; particle inflow gene gun [44], Helios gene gun system [45], and *Agrobacterium*-mediated transformation [46]. However, the recombinant proteins in the plants need 2-10% of the microbial frameworks for the delivery. Recently, the oral uptake of the plant-based therapeutic protein is said to be eliminating the need for cold storage. Besides, the freeze-dried plant cells expressing the pharmaceuticals are protected in the stomach from certain enzymes and acids as well as in the gastrointestinal tract which leads to expression stability [47] [48].
Biopharming is in the developing stage in Europe and still there is no human Plant Manufactured Pharmaceutical (PMP) approved for marketing authorization and commercialization to date. Many plant-based therapeutic proteins are either in the pre-clinical, clinical, or close to commercialization. Approximately 80% of all PMPs are in pre-clinical phase and phase I clinical trials, about 15 % are in phase II and around 5 % of the products have reached Phase III clinical stage. Europe represents around 10 % of the field trials for Biopharming and holds 19 % of Biopharming patents filed worldwide [49]. In 2019, Schillberg et al. reviewed and summarized the commercial potential of the plant expression system [50].
Protalix Biotherapeutics (Carmiel, Israel) produced the first pharmaceutical from plants (tobacco and rice), named *Taliglucerase alfa*, a drug used to treat Gaucher's Disease [51]. In parallel, a current good manufacturing practices (CGMP) factory has been working on biopharmaceutical production, for example, Greenovation Company produced Moss based therapeutics for Orphan diseases and it is in the preclinical trials [48].

However, there are some disadvantages of plants that produce biopharmaceuticals, where platform choice is case-specific and depends on a broad range of criteria. A broad range of plant species has been used for PM farming including *Arabidopsis*, banana, barley, carrot, flax, lettuce, maize, pea, peanut, pigeon pea, potato, rice, rape, safflower, spinach, soybean, sugar beet, sugar cane, tobacco, tomato, wheat, white clover, and white mustard [52]. *Arabidopsis* plants are mostly used as a model organism to study the actual gene expression, while the actual production is carried out in maize, potatoes, rice, flax or safflower, and tobacco. Clinical trials have been conducted with a smaller range of plants, the most relevant of which are maize, tobacco, rice, and safflower, and *Arabidopsis* [53].

4. Plant derived vaccines

The development of human vaccines produced in plants has also been supported by the large number of studies showing the efficacy of plant-derived vaccines for the prevention of diseases in other animals, culminating with the USDA approval of the Newcastle disease [10].
Plant-derived vaccines can be divided into two categories – those designed for veterinary use and those designed for medical use. The Newcastle disease vaccine for poultry was the first plant-derived pharmaceutical product to be approved, and there is a large body of both immunogenicity and challenge data to support the efficiency of such vaccines, including numerous clinical studies [54]. Several human clinical trials involving plant-derived subunit vaccines have also been reported. Recent studies have demonstrated the ability in vivo and vitro vaccine production in plant systems [55-58].
Edible vaccines are promising candidates because they are easy-to-store, cost-effective, easy-to-administer, and socio-cultural, especially for the poor developing countries. It simply introduces selected and desired genes into plants by molecular biology techniques and then induces these altered plants to manufacture the

encoded proteins. In 1998, the National Institute of Allergy and Infectious Diseases (NIAID) has proved that immunogenicity can be induced by an edible vaccine [24]. A variety of delivery systems have been developed. Initially thought to be useful only for preventing infectious diseases, it has also found application in the prevention of autoimmune diseases, birth control, cancer therapy, etc. The production of Edible vaccines is tuned mainly for animal diseases but eventually does not disclose the production for human diseases. There is a growing acceptance of transgenic crops in both industrial and developing countries [59].
To create an edible vaccine, the introduction of the selected desired gene into the plant is required, later on inducing these altered plants to manufacture the encoded proteins. This process is known as "transformation, and the altered plants are called transgenic plants." There are several approaches for vaccine expression in the plant, including; stable nuclear transformation, stable chloroplast transformation, or transient expression and stable transformation by hydroponically grown plants. The transient expression has shown a great advantage in the production of rapid response proteins and vaccines during the Ebola outbreak in 2014 [60]. In 2014, a study was performed in which an anti-Ebola antibody cocktail was produced in the tobacco plants [61].
Like conventional subunit vaccines, edible vaccines are composed of antigenic proteins and are devoid of pathogenic genes. Thus, they have no way of establishing infection, assuring its safety, especially in immune-compromised patients [62]. The conventional subunit vaccines are complicated to produce, they are expensive and technology-intensive, require refrigeration and produce a poor mucosal response, and need an additional purification process. In contrast, edible vaccines enhance compliance, especially in children, and because of oral administration, would eliminate the need for trained medical personnel. Edible vaccine production is highly efficient and can be easily scaled up. They are cheaper and exhibit good genetic stability. They are heat-stable; do not require cold-chain maintenance; can be stored near the site of use, eliminating long-distance transportation. Non-requirement of syringes and needles also increases the chances of infection [63]. Fear of contamination with animal viruses - like the mad cow disease, which is a threat in vaccines manufactured from cultured mammalian cells is eliminated, because plant viruses do not infect humans. By using edible vaccines, the mucosal and systemic immunity is activated, as they come in contact with the digestive tract lining. This dual effect would provide first-line defense against pathogens invading through the mucosa, like Mycobacterium tuberculosis and agents causing diarrhea, pneumonia, STDs, HIV, etc. [64]. Edible vaccines are suitable against neglected/rare diseases like dengue, hookworm, and rabies by using model organisms such as banana, potato, tomato, lettuce, rice, etc. However, till now, two products have been licensed, including; scFV monoclonal antibody for the production of HBV vaccine and NDV vaccine approved by the US Department of Agriculture (USDA) [24]. Edible vaccines are currently being developed for several human and animal diseases, including measles, cholera, foot and mouth disease , and hepatitis B, C and E [65]. Interestingly, several studies have demonstrated the production of viral antigens or anti-viral proteins that could be used as emergency vaccines for COVID-19 [66-68]. Recently, several companies started developing potential plant-based vaccines for COVID-19, such as; Medicago, iBio and Kentucky BioProcessing [69].

4.1. " Second-Generation" Edible Vaccines

Several clinical trials have reported the potentiality of edible vaccines, in the prevention of various diseases. Antigen expression in plants was successfully studied in tobacco and potato, rabies virus G-protein in tomato, HBs AG (hepatitis) in tobacco and potato, Norwalk virus (gastroenteritis) in tobacco and potato, CT-B (Vibrio cholera) in potato [70]. One of the multi-component vaccines is Poly-N-acetyl glucosamine (PNAG), a beta-1-6 linked surface polysaccharide, which is expressed by a broad range of bacterial pathogens, including *Staphylococcus aureus*, *Escherichia coli*, and *Yersinia pestis*. It has a high potential to be a widely protective vaccine against a diverse array of pathogens. In 2018, a study has shown another example of the multi-component vaccine in which transplastomic plants could yield multi-component vaccine production against cysticercosis [71].

4.2. The Prospective of Plant Derived Pharmaceuticals

As plant biotechnology develops, the restrictions involved in the production of the plant made proteins are being eased. The glycol-engineering results in the plant field demonstrate that plants may have an added advantage as they are very flexible and subunit vaccines containing any glycan structure can be produced, with potentially very high product homogeneity. This would allow the commercial production of many natives (humanized) glycol-vaccines in many species [72]. However, now the technology is only starting to be implemented due to investment by big pharmaceutical companies. Once the infrastructure is in place for these

ventures, it may become more common for plant systems to be the preferred method of protein production in the future. Transgenic technology has improved in the last 20 years, with many more species being routinely transformed with simpler and a range of different methods [73]. While there has been a lot of work done on enhancing the transformation capabilities of plants, with many vectors being designed with various advantages and disadvantages, very little research has been done into optimizing the plants themselves. As plant bio-factories become more common, the next important step is to optimize the plants used, and identify the conditions that suit the expression of specific proteins [74]. In 2017, a study has shown that the production of recombinant protein in root cultures could improve the quality and quantity of proteins without downstream-processing. Related to this, in 2020, a study was performed to discuss the application of hairy roots culture for protein production [75].

The idea behind the edible plant vaccines was the ability to vaccinate someone by eating a piece of fruit or vegetable. However, recombinant plant proteins are only used after being highly purified. It is noted that the raw edible vaccines are an unfeasible technology for human vaccines and therapeutics, it may not be necessary to fully isolate the target protein from plant material. Therefore, a dried and ground plant material would be suitable for the oral delivery of vaccines and some therapeutics, as well as batch testing and analysis. In this case, a balance would have to be done between achieving the correct dose of the vaccine while reducing the amount of any detrimental compounds. This could be greatly helped by optimizing the host plant's characteristics with regard to the number of detrimental metabolites it contains. Such oral vaccines could be produced cheaply and easily for use in developing nations [72]. This would also be an excellent option for the production of veterinary vaccines where recombinant feed could contain vaccine antigens. If yields can be better standardized, then there is potential for the delivery of therapeutics in unprocessed plant material, especially for veterinary purposes or for products where the dosage has a wide active range [76]. However, that would not be a realistic option until the whole and partially purified products are on the market and shown to be safe and effective. It is likely that partially purified vaccines will first be introduced for veterinary purposes and then progress to humans, once the technology gains acceptance. It is important that innovation continues in the field of plant-made pharmaceuticals and vaccines to confirm the technology's potential to become a major platform for recombinant protein productions and that the regulatory frameworks, responsible for the approval of plant therapeutics, relax their expectations and provide more opportunities for companies that started their clinical trials [77].

5. Conclusion

Production of pharmaceuticals in plants for therapeutic purposes shows great promise, with some PMPs in clinical trials and many others under investigation. Plant production systems are easily expanded and typically provide a lower cost of production relative to the cell culture systems currently used to produce biological therapeutics. Regulation agencies in Europe and the United States are actively developing the agronomic and manufacturing regulations needed to ensure the safety, consistency, and potency of plant-made pharmaceuticals. With this increased availability and potentially lower cost, more patients will be able to receive the drugs they need.

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