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Plant-Based Recombinant Vaccine: Fact or Fiction?

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Abstract

In the era of recombinant DNA technology, production of recombinant vaccines in green plants has emerged as an effective approach addressing the problems of traditional vaccine production. Various antigens expressed in different plant species have been so far tested for the production of efficient oral vaccines against human and livestock diseases. However, recombinant vaccines have not yet found a prominent place in pharmaceutical market. There are still many challenges to be addressed to pave the road for commercial production of plant-based recombinant vaccines. Regarding increasing growth in laboratory studies and field trials for development of plant-based vaccines, this review paper provides a comprehensive overview on the topic of plant-derived vaccines and related issues. **[GMJ.2017;6(4):268-80] DOI:10.22086/gmj.v6i3.792**

Keywords: Recombinant; Vaccine; Plant

Introduction

Plant molecular farming is the science and, more exactly, the art of producing recombinant proteins or secondary metabolites in plants. This technology begins with genetic modification of the plant with the gene of interest and involves the whole process of growing, harvesting, and downstream processing of the genetically modified (GM) plants to extract and purify the recombinant protein [1]. Therefore, the principles of producing plant-based pharmaceuticals are quite similar to those used in GM crop production. The genetic information required for the production of therapeutic proteins is carried on a DNA molecule. By application of genetic engineering tools, this DNA molecule is introduced into the plant cell and is integrated in its genome. This process is called genetic transformation. The foreign gene integrated in the plant genome is then transcribed and translated to the recombinant protein of interest by protein-making machinery of the host plant. This process explains the way the plant acts as a bioreactor, producing large amounts of biologically active therapeutic proteins [2]. Plant-derived pharmaceuticals (PDPs) are proteins or organic compounds produced in plants via recombinant DNA technology that 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 the production of therapeutic products is an emerging field of biotechnology with high economic potential [3]. The application of green plants

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as bioreactors for the production of therapeutics stems from their utility in terms of economic, safety, and quality considerations. The contributing factors for the increasing growth in the biopharmaceuticals market include the widespread prevalence of chronic disease, technological advancements in biopharmaceuticals, and increasing R&D investments. Furthermore, the potential ability of therapeutic recombinant proteins to target a special tissue helps in the treatment of various diseases. Biopharmaceuticals such as recombinant vaccines, hormones, and antisense drugs can heal the diseases that are otherwise difficult to cure. These properties of recombinant therapeutic proteins have paved the road for the increasing growth of the biopharmaceuticals market. According to official statistics, global biopharmaceuticals market was valued at US $162 billion in 2014 and is expected to reach US $278 billion by 2020 at a CAGR (compound annual growth rate) of 9.4% during the forecast period [4]. Recombinant vaccines constitute a major portion of PDPs. A plant-based vaccine is indeed a recombinant antigen produced in an herbal host by means of recombinant DNA technology and genetic engineering tools to serve as elicitors of protective immunity throughout the administration by distinct routes [4]. Although vaccination with conventional vaccines has proved to be an effective practice in the prevention of diseases, there still is disagreement over its use. Some of the documented adverse effects of the elements and substances used in vaccine serums include blood disorders, autoimmune diseases, cerebral palsy, brain damage, paralysis, neurological impairment, monkey fever, autism, mental retardation, premature aging, and so on. Thus, there is an urgent need to find an alternative to the present vaccines. This alternative can be the development of plant vaccines [5]. Considering the 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 [6]. For many years, recombinant vaccines were exclusively expressed in expensive systems such as yeast or mammalian cells. These platforms, although effective in production rate, suffer from high production costs and the risks of contamination by human pathogens. On the other hand, recombinant vaccine production in bacterial cells, though simple and cost-effective, was not scalable to commercial level because of improper folding of eukaryotic peptides and occurrence of inclusion bodies in bacterial cells [7]. Genetic transformation of higher plants was a milestone in the field of recombinant vaccine production. Scientific groups began to produce transgenic plants that were able to induce an immune response in the body when administered through the oral or parenteral route. 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 [8]. This work was soon followed by the expression of the hepatitis B surface antigen (HbsAg) [9], the Escherichia coli heat-labile enterotoxin responsible for diarrhea, and the rabies virus glycoprotein [10] in plant species. Proteins produced in these plants induced the synthesis of antigen-specific mucosal immunoglobulin A (IgA) and serum immunoglobulin G (IgG) when delivered orally to mice and humans. Compared with 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 because of the lack of any mammalian pathogens. With the increasing demand for recombinant therapeutics, together with the high costs and inefficiency of the existing production systems such as bacteria, yeast, and mammalian cell culture, green plants have gained much attention as a production platform among academic and commercial bodies [11]. The cost of vaccine production in plant systems is comparable with that of microbial bioreactors and is 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 they are naturally free of microbial toxins and human and animal pathogens [12]. 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 [6]. Another important advantage of recombinant plant-based vaccines lies in the flexibility to respond to fluctuations in demand. This is realized by the so-called transient gene expression technique; in which a large quantity of the antigen of interest is produced in plant tissue in a short time. Vaccine Company Medicago Inc, Canada, is the pioneer in practical use of transient gene expression for the production of recombinant vaccines in a market-sensitive manner. The researchers of this company have established a system for quick production of recombinant vaccine in a short period. The mechanism and features of transient gene expression approach will be discussed in detail in the next sections. A schematic diagram representing successive steps for production of plant-based recombinant vaccines is depicted in (Figure-1).

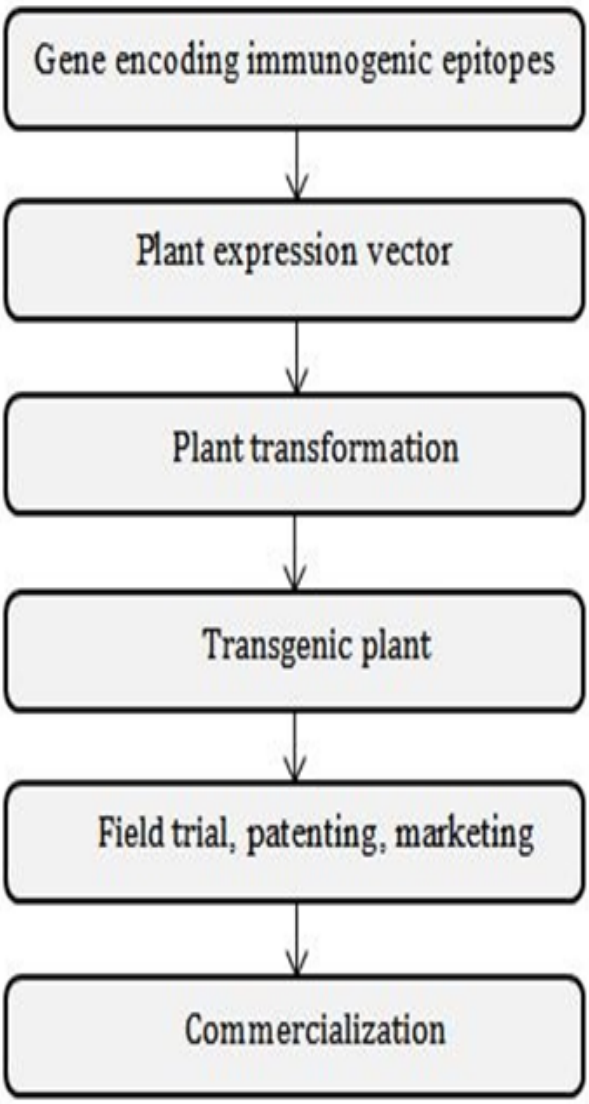

Figure 1. The procedure of recombinant vaccine production in plant systems

Search Strategies

This study follows a qualitative approach to investigate various aspects of production of recombinant vaccines in plant hosts. In this regard, a thorough review was performed over a large number of papers published since the first report about expression of a recombinant antigen in a plant species (1992). For this purpose, authentic papers published in scientific journals indexed in PubMed, Elsevier, Scopus and so on, were studied to extract relevant information about various dominions of plant-based recombinant vaccines.

Plant Choice

Adoption of an appropriate plant species is a crucial factor for the success of recombinant vaccine development. Indeed, the choice of plant species is the first step in the production of every plant-based vaccine. But what is the best plant species for molecular farming? Is there a single species amenable to produce every type of recombinant vaccine? What are the main criteria affecting decision making about the selection of plant species for the development of recombinant vaccines? In general, adoption of a plant species depends on both economic and technical factors, including total biomass, ease of transportation, value of the recombinant protein itself, scale-up costs, availability of labor, land area required, length of production cycle, and costs of downstream processing. The suitable host should, in addition to economic consideration, be amenable to transformation and regeneration. Thus, the best host plant for a production of recombinant protein should be determined empirically

based on a number of influencing factors [13]. Many species can be adopted for the production of recombinant vaccines, with tobacco being the most widely used host plant to date. The advantages of the leafy crop, tobacco, include the high biomass yield, all-year-round growth and harvesting, availability of large-scale infrastructure for processing, availability of well-established transformation protocols, and ease of stable transformation either by cocultivation with *Agrobacterium tumefaciens* or transiently by infiltration with transgenic agrobacteria or transfection with viral vectors [14-17]. 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. However, tobacco contains high content of alkaloids and toxic compounds, which should be removed during purification process; this practice enhances the costs of downstream processing, creating an additional hurdle for commercialization of recombinant vaccine production [1]. Other examples of leafy crops used for the production of recombinant vaccines include alfalfa [18], white clover [19], spinach [20, 21], and lettuce [9]. Tomato (*Solanum lycopersicum*) 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 [10]. Other examples include cholera toxin B subunit [22] respiratory syncytial virus-F protein [23], toxin coregulated pilus subunit A (TCPA) of *Vibrio cholerae* [24], and so on. Other examples of fruits and vegetables used for antigen expression include potato [25, 26], lettuce [27], and carrot [28]. 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 the production of recombinant vaccines [3]. 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 copurify with the recombinant protein during downstream processing [29]. 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 [30]. Green microalgae are considered as efficient cell factories for the development of recombinant vaccines. Microalgae represent the advantages of both prokaryote and eukaryote organisms. Microalgae are unicellular organisms with very fast growth habit, which produce large volume of biomass in a short period (prokaryotic feature). Moreover, because of their eukaryotic nature, they are able to process long eukaryotic peptides with exact folding and accurate posttranscriptional modification [31]. Examples of recombinant vaccine produced in microalgae include expression of foot-and-mouth disease virus (FMDV) VP1 antigen in *Chlamydomonas reinhardtii* [31], fusion protein containing the VP1 gene and the cholera toxin B subunit [32], and syndrome virus protein 28 (VP28) [33] in chloroplast genome of the same species. Selecting a plant platform for the production of recombinant vaccines depends on various considerations, with the first one being vaccine delivery form. Recombinant vaccines can be expressed in both fresh tissue, such as mature plant leaves and germinating seedlings, and dry materials such as the cereals [34]. Hairy root culture (Figure-2) is another choice because the system enables the recombinant

vaccine to be secreted into culture medium [35]. The plant species selected as expression system should possess optimum antigen expression, allowing for cost-effective production, along with the possibility of manufacturing it into a practical form for oral delivery.

Transformation Strategies

Recombinant vaccines can be expressed by both stable and transient transformation in plants. Stable transformation is the most widespread approach for production of transgenic plants expressing the recombinant antigen. In this approach, the gene encoding the antigen protein is transferred into nuclear or plastid genome by *Agrobacterium* or biolistic method [36]. In gene transfer mediated by *Agrobacterium*, the gene of interest is inserted into the T-region of a disarmed Ti plasmid of *A. tumefaciens*. *Agrobacterium*-mediated transformation works well for dicotyledon plants such as alfalfa, tobacco, and tomato [14]. A number of important crops (e.g., cereals) are recalcitrant to *Agrobacterium* transformation, and hence, a biolistic method is used for these plants [6]. In this approach, DNA-coated gold particles are propelled into plant cells using compressed helium gas, which then become incorporated into chromosomal DNA. The biolistic method usually results in higher-copy-number plants compared with those generated by *Agrobacterium*, which can enhance expression. However, excessive copy numbers or very high-level expression of nuclear genes can cause gene silencing, thereby resulting in low protein accumulation. Thus, it is important to select transgenic lines that carry only between one and three copies of the transgene [3]. In transient approach, 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,

Figure 2. Hairy root clone expressing anti-newcastle disease virus (NDV) recombinant vaccine [35]

Figure 3. Agroinfiltration assay for transient expression of VP1 epitopes in tobacco leaf (left); infiltration patches on the leaves (right) [41]

never become integrated into the plant genome and hence are only expressed by the generation of infected cells [37]. Transient expression of immunogenic proteins has been applied to tobacco, black-eyed beans, and spinach [38]. Transient expression of foreign proteins can also be mediated by *A. tumefaciens* by a technique called agroinfiltration. Agroinfiltration (Figure-3) involves the injection or vacuum infiltration of plants' leaves with a suspension of bacteria harboring the antigen of interest. Owing to its advantages, agroinfiltration has gained numerous applications and hence, has been used for the investigation of molecular processes and production of interesting molecules of monoclonal antibodies [39], and antigens of human [40] and livestock [41] pathogens. In addition, 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 [42].

Enhancing Antigen Expression Level in Plants

Insufficient rate of recombinant protein expression has always been a major drawback for commercialization of plant-based subunit vaccines [43]. As noted elsewhere [15], significant enhancement of transgene expression in plant tissue will be a milestone in the production of plant-based recombinant vaccines [15, 36]. Several approaches, including codon optimization, the use of strong plant promoters, and untranslated leader sequences, have been prepped and tested to elevate transgene expression in plant species [44]. Codon optimization is regarded as an effective tool to enhance the rate of transgene expression in transgenic plants [45]. It is documented that codon optimization is able to increase expression level in nuclear transformation as high as 5-fold [46] or up to 80-fold in chloroplast transformation [7]. Moreover, presence of rare codons in some organisms significantly attenuates efficacy of translation machinery in transgenic plants [47]. This fact has encouraged many researchers to use synthetic gene with optimized codon sequence [41, 43]. Presence of leader sequence at 5′ untranslated region of the transgene is another option to promote expression level. Kozak leader sequence (GCCACC) is a ribosome-binding site (RBS) that plays a remarkable role in the enhancement of translation efficiency in plant hosts [48]. The upstream leader of the Tobacco Mosaic Virus (TMV) called Ω sequence is another untranslated region that significantly increases translational power in higher plants. The CAA region within Ω sequence is the main cause of translational enhancement and acts as a binding site for HSP101 heat shock protein; the latter is necessary for translation improvement [49]. In some cases, signal peptides such as SEKDEL sequence (Ser-Glu-Lys-Asp-Glu-Leu) have been used to target the antigen in to the endoplasmic reticulum (ER), where necessary enzymes and cellular machinery for proper folding are present[50]. By addition of ER signals to transgene, high level of antigen expression has been observed in a number of studies [43, 51, 52]. The ER signals are often attached to 3′ end of the transgene just before stop codon [41]. Chloroplast transformation is a well-known strategy to improve the accumulation of foreign protein in plant tissues. This technique, which is called cpDNA transformation, includes the integration of the transgene into the circular chloroplast DNA (cpDNA) that is present in multiple copies in plant cells. The cpDNA transformation offers numerous advantages: the cpDNA molecule is completely sequenced in a number of important plants and is present up to 10,000 copies per cell. Moreover, chloroplasts can properly process eukaryotic proteins, including correct folding and disulfide bridges [53]. Integration into cpDNA has two important advantages; first, the foreign sequence is targeted to a precise cpDNA site by homologous recombination. Such integration addresses the problem of variability in gene expression and gene silencing, which is a common phenomenon in nuclear transformation. The second advantage is

the enhanced accumulation of the recombinant antigen. Accumulation of recombinant protein in chloroplast engineering is significantly higher than that of nuclear transformation [54].

Oral Delivery

Induction of mucosal immunity is an important aspect of plant-based vaccines and even a major competitive advantage of such vaccines in global market of pharmaceutical products [36]. Infectious pathogens usually enter the body through mucosal membranes. Mucosal immunity is well induced if the vaccine is directly delivered to mucosal surfaces [55]. The main problem limiting the use of recombinant vaccines expressed in yeast, bacteria, and mammalian cells is that the recombinant proteins may be degraded after ingestion and that some immunogens may not be recognized efficiently at mucosal immune effector sites in the gut. In contrast, plant cell wall provides a shield around the protein, inhibiting early degradation of the recombinant protein [41]. 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 [56]. 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. Similar to liposomes and microcapsules, the plant cell wall allows the gradual release of the antigen onto the vast surface area of the lower digestive tract [34].

Commercialization Challenges

Recombinant vaccines expressed in plant hosts are free from human pathogen contaminants. Moreover, plant DNA does not interact with the animal DNA, and plant viral recombinants do not invade mammalian cells. However, there are still some concerns hindering commercialization of plant-based vaccines. A major concern is the risk of GM-pollen escape to natural habitats, which may cause harmful influences on biodiversity. To address this challenge, some pollen containment approaches based on the establishment of different forms of male sterility have been developed [36]. An alternative approach would be engineering epitope genes into the cpDNA, which is not transmitted to the sexual progeny through the pollen grains [53]. 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 plant-based recombinant vaccines is the presence of antibiotic resistance genes used as selective marker in the transgenic plants. Some techniques have been developed to generate marker-free GM plants. This is the case for both nuclear and chloroplast transformation [56].Despite numerous advantages of plant-based recombinant vaccines, most of pharmaceutical companies are still reluctant to make considerable investment in the field of plant-derived vaccine development for human and animal diseases. This hesitation stems mainly from the concern about the potential for significant return on investment, market acceptance, lack of governmental support, problems in regulatory processes, lack of personnel with sufficient expertise, and so on [57]. From business viewpoint, commercialization of recombinant vaccines is a type of new product development [58].

In management literature, new product development (NPD) refers to the process of bringing a new product into market. Additionally, NPD is defined as the transformation of a market opportunity into a product available for sale [59]. NPD can be applicable for both tangible and intangible products [60]. Regarding plant-based recombinant vaccines, tangible aspect of NPD is more apparent. For success in commercialization of plant-based vaccines, a good understanding of customer demands, the competitive environment, and the nature of the market represent the top required factors for the success of a new product [61]. Customer acceptance of a new product depends on cost, delivery, and, more notably, the quality of the new product

[62]. Therefore, a new biotech-based product should present higher quality and more reasonable cost to get general acceptance [63]. Considering plant-based recombinant vaccines, they should be of higher quality and lower cost compared with traditional vaccines to find their way in the marketplace [64]. Biotech companies always try to develop effective strategies to fulfill customers' demands and increase their market share by a regular development of new products [65]. As mentioned elsewhere, the application of best marketing activities and addressing the public concerns are the main concerns for the management of NPD process in biotechnology companies [66].

To understand the reasons behind the lack of sufficient number of recombinant vaccines in marketplace, it is necessary to consider the concept of supply chain. Supply chain is defined as an integrated complex of companies, individuals, activities, information, and resources delivering a product or service from supplier to customer [67]. Indeed, supply chain includes the transformation of raw materials into a finished product that is sold to the customer [68]. In this regard, the question may be: what is the exact raw material for the production of recombinant vaccines? Is it the plant host or the gene of interest? This is an example of fundamental difference between biotech and ordinary products supply chain. Moreover, in routine supply chains, manufacture of a product is only the first step, and other activities such marketing, delivery, customer management, and so forth play a critical role in bringing a product into market [69].

For recombinant vaccine production, the majority of the practices are still stopped in the production phase [70], and no effective endeavors are being made to introduce recombinant products to the public [71]. As mentioned elsewhere, recombinant products will not achieve public acceptance unless the way be paved for marketing of such new products [72]. Recombinant products are required to be labeled in European countries. The European Union regulatory framework has slowed down the approval process of GM crops, which puts more constraint on market success of recombinant products [73]. In such an unbalanced battle between GM products and non-GM products, more attempts are required to bring recombinant vaccines into the markets [74]. Encouraging results have been always reported about applicability of plant-based recombinant vaccines for combating human diseases [75]. In 2016 alone, for instance, numerous papers have been published on the successful expression of recombinant vaccine in plants against influenza [76], malaria [77-79], porcine cysticercosis [80], Newcastle disease [35], Rift Valley fever [81], as well as others. These examples show the great potential of recombinant vaccine technology to produce sufficient amount of medicines for fighting infectious diseases. However, as mentioned before, more attempts are needed to bring the plant-based vaccine in the global market.

Discussion

In this paper, we reviewed the history of development of plant-based recombinant vaccines and described the latest progress made by researchers in this field. The main goal of this review paper was to elucidate whether the issue of plant-based vaccine production is really a practical field of investigation or it is merely a fantastic topic. Simply speaking, although the advantages of plant-based vaccine development might have been exaggerated, the numerous examples of successful induction of immune response in laboratory animals indicates that the antigens expressed in plant hosts can really be used as effective vaccines. This claim is strongly supported by the development of some plant-based recombinant vaccines that successfully passed the trial phases and are now commercially available in pharmaceutical market. However, as indicated in the previous section, a number of problems should be addressed to put the recombinant vaccines in the pharmaceutical market [56-72].

Conclusion

Considering the recent progress in the field of genetic transformation of higher plants, application of plant-based platforms can be an effective and affordable approach for

production of a large variety of recombinant vaccines against various diseases. Since the pioneer work of Curtiss and Cardineau [8], numerous studies have been conducted across the world to demonstrate the feasibility of the production of plant-based vaccines. However, different issues, including high expression levels, product quality, downstream process costs, regulatory framework, efficacy, and safety should be addressed to observe commercial recombinant vaccines in pharmaceutical market. Moreover, a substantial part of the research in the field of recombinant vaccine production is limited to laboratory assays in tobacco, which is not an edible plant and, because of the presence of 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 the production of a plant-based recombinant vaccine.

Conflict of Interest

None declared.

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