Review Article

Plant-Based Vaccines: Production and Challenges

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Plant-based vaccine technologies involve the integration of the desired genes encoding the antigen protein for specific disease into the genome of plant tissues by various methods. *Agrobacterium*-mediated gene transfer and transformation via genetically modified plant virus are the common methods that have been used to produce effective vaccines. Nevertheless, with the advancement of science and technology, new approaches have been developed to increase the efficiency of former methods such as biolistic, electroporation, agroinfiltration, sonication, and polyethylene glycol treatment. Even though plant-based vaccines provide many benefits to the vaccine industry, there are still challenges that limit the rate of successful production of these third-generation vaccines. Even with all the limitations, continuous efforts are still ongoing in order to produce efficient vaccine for many human and animals related diseases owing to its great potentials. This paper reviews the existing conventional methods as well as the development efforts by researchers in order to improve the production of plant-based vaccines. Several challenges encountered during and after the production process were also discussed.

1. Introduction

Vaccines help in stimulating the antibodies production in human and animals and provide immune protection against several diseases [1]. However, the unavailability of vaccines for the treatment of fatal diseases has caused problems and driven global attention towards production of safer, easier, and more effective vaccines. Generally, there are three types of vaccine production methods, namely, the egg-based vaccines, cell-based vaccines, and vaccines produced using investigational-manufacturing systems. The most common example of egg-based vaccine is the influenza vaccine produced in 9-to-12-day-old embryonated eggs [2, 3]. This conventional method has been applied for over 60 years and it involves the injection of virus particles into the eggs and further incubation for several days to allow the replication of virus particles. In order to produce a vaccine, the antigen isolated from the purification process of the eggs containing

vaccine virus particle would undergo additional procedures. However, the selection of most appropriate influenza virus strains to be replicated for vaccine production remains to be the main limitation in this method as not all strains of influenza virus are able to replicate in embryonated eggs, hence affecting the amount of vaccine produced in the eggs [2]. Apart from that, a large number of eggs are required to produce a vaccine while the regulatory approval for vaccines produced from egg-based method is rather time-consuming [2, 4]. Meanwhile, the main limitation in producing the cell-based vaccines would be the requirement of high-priced fermentation facilities. With various limitations in the former two conventional methods, particularly in relation to time, the expensive manufacturing process due to the need of cold storage for the temperature-sensitive vaccines [5], and the risk of unwanted immune response and developing the disease, investigational-manufacturing systems, which utilize the biological systems such as plant, insect cells, or bacteria

culture to manufacture vaccines have recently gained the attention of researchers. Among these, plant-based vaccine production has received particular attention due to the numerous advantages it may offer.

The attempt to produce vaccines in plants was made by Hiatt and coworkers in 1989 [6]. The concept of utilizing transgenic plants to produce and deliver subunit vaccines was introduced by Dr. Arntzen and his colleagues and proved that this concept can overwhelm the limitations in traditional vaccine production [6]. The first subunit vaccine was produced by them in tobacco plants by expressing surface protein antigen of *Streptococcus mutants*. They also initiated the production of hepatitis B and heat-labile toxin B subunit in potato tubers as well as potato plants. In 1998, it was proven, for the first time, by National Institute of Allergy and Infectious Diseases (NIAID) that significant immunogenicity can be induced safely by an edible vaccine [6], utilizing the concept of plants as bioreactor. Due to the fact that the plant-based vaccine is easy to handle as it does not require complicated storage and its production is cost-effective and easy to scale up for large production, this method may provide a cheaper alternative for vaccine production [5, 7–10]. Moreover, plant-based edible vaccines produced through this method are able to provide a needleless, convenient, and easy route of administration [9–11]. Among the plants that have been commonly used as bioreactor are tobacco, potato, tomato, corn, and rice. To date, there are many transgenic plants that have been used to produce four different types of vaccines: bacterial vaccines, viral vaccines, parasite vaccines, and immunocontraceptive vaccines [9].

There are several plant-based vaccines that have been produced, with some of them being currently at the clinical trial phase. Among them, the most common types of vaccines are against virus and bacteria that cause fatal illness in human and animals and usually *Nicotiana* plants are utilized as the bioreactor. However, to date, only two products have been licenced: (a) plant made scFV mAB used in the production of a recombinant HBV vaccine in Cuba and (b) Newcastle disease virus (NDV) vaccine for poultry approved by the US Department of Agriculture (USDA) [12]. There is no plant-based vaccine that has received the license from US Food and Drug Administration (FDA). This is due to the fact that plant-based vaccines are classified under the genetically modified crop category [13]. In view of this exciting yet challenging research, the first part of this paper focuses on the conventional and refined expression technologies for improved plant-based vaccines production, while the latter part discusses challenges encountered during and after the production process.

2. Production of Plant-Based Vaccines

Plant-based vaccine production mainly involves the integration of transgene into the plant cells. The target sequence of the selected antigen is integrated with the vector before being transferred into the expression system. The transgene can then be expressed in the plants either through a stable transformation system or through transient transformation system, depending on the location where the transgene has been inserted in the cells. Stable transformation system can be achieved through nuclear or plastid integration [14]. It is called stable or permanent due to the permanent changes occurring in recipient cells' genetics as the target transgene is integrated into the genome of host plant cells [15]. Biolistic and genetically modified *Agrobacterium* strain can lead to the formation of stable transfection. However, as *Agrobacterium tumefaciens* is not infecting many plant species naturally, it limits the application of *Agrobacterium* strain for stable transformation of the desired gene. Generally, stably transgenic plant cells produce a lower amount of subunit antigen, in the range of 0.01 to 0.30% of total soluble plant protein.

On the other hand, transient transformation system involves the production of desired protein or antigen soon after the heterologous gene resides transiently in the host cells [14, 16]. The transgene is not incorporated into the genome of the plant cells. In this plant expression system, the regeneration of whole plant is not required and the frequency of its occurrence is higher. These characteristics overcome the pitfalls related to the stable integration [14]. Two most commonly used methods that would achieve transient expression of a desired protein in plants are the *Agrobacterium*-mediated transformation of genetically modified plant virus and particle bombardment [14].

2.1. Direct Gene Delivery Method. As mentioned earlier, there are several methods that can be used to produce plant-based vaccines. Basically, these methods are divided into two categories, which are direct and indirect gene delivery [16, 17]. The direct gene delivery method simply means the direct introduction of DNA or RNA into the plant cells [12]. In this section, the most common direct gene delivery approach, biolistic method, will be further discussed.

Biolistic method is a vector-independent method and it is also known as gene gun or microprojectile bombardment method [12]. This is an alternative method of gene transfer for nuclear transformation if *Agrobacterium*-mediated transformation is not feasible [6, 18, 19]. It involves the use of gold or tungsten as microcarrier to coat the DNA [6, 20]. The coated DNA will then be placed on top of macrocarrier, inserted into gene gun, and subjected to high pressure of helium gas [6, 20]. Due to the high pressure, the coated DNA will travel at a high speed within a vacuum and penetrate into the cells of targeted plant [21]. The advantages of this method are that it forms a stable integration of the transgene into the plant genome and it can be applied to transfer foreign DNA into a variety of types of plant host species as well as various cell types [14]. There is no vector requirement for this method and it will aid in cotransformation [14]. However, it requires a costly particle gun device, it is labour intensive, and it can cause severe damage to the plant tissues [16, 20].

Biolistic method can be used to achieve two types of antigen expression in the transgenic plants: nuclear and chloroplast transformation. Nuclear transformation is done by integrating the desired gene into the nucleus of the plant cells via nonhomologous recombination [22, 23]. The transgene might be inserted at the same locus or different loci to create the stable transgenic plants [22]. Even though the plants can inherit the transgene to the offspring, nuclear transgenic

plants show a low expression level of antigens which lead to the requirement of a huge quantity of plant material to produce the right dose of administration and it might cause pleiotropic and position effects due to the random integration of the transgene [23–25].

A fascinating alternative to nuclear transformation with the aim to increase the yield of recombinant protein production from a single transformation step is known as chloroplast transformation [6, 17, 26]. The formation of chloroplast transgenic plants involves the use of biolistic process, which will deliver the desired DNA into chloroplasts, followed by the integration of gene of interest into the chloroplast genome from plastid transformation vector at flanking sequence via homologous recombination [18, 26]. The advantages of chloroplast transformation compared to the nuclear transformation are the ability to eliminate gene silencing effect, a rapid and low cost production due to its high copy number in a plant cell, its potential to express multiple genes in plastids and less technical work, natural transgene containment, and ensuring a site-specific insertion of the transgene in the chloroplast genome [6, 17, 25, 27, 28]. As plastid will be generally maternally inherited to the next offspring, gene flow through pollination to the transgenic plant's weedy or wild relatives in the same environmental location can be prevented as the transgene is inserted and expressed in the plastid genome [29]. However, as no glycosylation process happens in plastids, thus this method is not an option for production of functional heterologous proteins that require a complex posttranslational modification.

Among nuclear and chloroplast transformation, most of the recently reported plant-based vaccines are produced through chloroplast transformation. Some examples of vaccines that were derived from chloroplast to fight against bacterial diseases are cholera, Lyme disease, anthrax, tetanus, and plague, while vaccines to fight against viral diseases are rotavirus and canine parvovirus (CPV) [26]. Production of most of these vaccines utilized tobacco as the model plant. The cholera toxin B subunit, *Bacillus anthracis* protective antigen, and tetanus toxin Fragment C genes were all expressed utilizing the transgenic tobacco model [30–32]. Similarly, a protective peptide, namely, 2L21, which prevents dogs from CPV infection, was also successfully expressed in the tobacco model [33]. Some plant derived vaccines were also further tested in animal models to validate their efficacy. For instance, results showed that mice immunized with the chloroplast-derived anthrax vaccine survived through the anthrax toxin challenge [34] while mucosal immunisation of mice with the chloroplast-derived tetanus vaccine increased the antibodies against tetanus in the body, indicating the safe use of the vaccine applied through nasal or oral route [32].

Apart from tobacco model, two plant-based vaccines against dengue and rabies viruses have been reported to be produced in *Lactuca sativa* (lettuce) and *Zea mays*, respectively, through biolistic method. The dengue virus vaccine targeting dengue-3 serotype polyprotein (DENV3prM/E) was produced in *Lactuca sativa* via chloroplast transformation [35]. Results showed that *L. sativa* expressed polyprotein of the antigen in different forms as monomers, heterodimers, or multimers in Western blot. The plant grew normally and the transgenes were inherited by the next progeny without any segregation. In another example, the rabies vaccine was produced in *Zea mays* whereby the embryogenic callus was first transformed with the pregenerated construct with a constitutive promoter from cauliflower mosaic virus (CaMV) by biolistics, and the regenerated plants were grown in a greenhouse [36]. Then, the transgenic *Zea mays* expressing the rabies virus glycoprotein was fed to mice through oral route. Results showed that the treatment protected the animals from the rabies virus challenge, conferring the beneficial effect of the plant-based vaccine as a potent oral immunogen.

Based on the current evidences, antigen expression through chloroplast transformation confers several benefits. First of all, the introduced genes were inherited stably in subsequent generations ensuring the continuous supply of the source [30]. Furthermore, high yield of antigens in the chloroplasts would lessen the amount of plant material required for vaccination, and this would make the encapsulation of freeze-dried material or pill formation easier [33]. Even though production of plant-based vaccine from chloroplast transformation had been identified as an alternative method to overcome the weakness in nuclear transformation, more studies need to be carried out. This is due to the fact that chloroplast transformation method is yet to be applied on many plant species apart from tobacco plant. In addition, it is difficult to create plants that will have uniformly transformed plastid (homoplasmic). Due to the later limitation, generation of transplastomic plants that are genetically stable is hindered.

2.2. Indirect Gene Delivery Methods.

Despite using direct gene delivery method, indirect gene delivery methods show more significant efficacy in vaccines production as indirect gene delivery involves the utilization of plant bacteria, particularly the *Agrobacterium* species and plant viruses, which naturally infect the plant cells and are able to integrate the gene of interest into plant genome [16].

2.2.1. Agrobacterium-Mediated Gene Transfer.

Agrobacterium is a Gram-negative soil pathogenic bacterium that naturally will infect the plants and transfer their genes (T-DNA) to the nucleus of the plant cells [17, 19]. Two strains of *Agrobacterium* species that have been commonly used as a biological vector are *Agrobacterium tumefaciens* (*A. tumefaciens*) and *Agrobacterium rhizogenes* (*A. rhizogenes*). The main difference between these two species is the plasmid that they carry. *A. tumefaciens* carries tumour-inducing plasmid (Ti-plasmid), while *A. rhizogenes* carries root-inducing plasmid (Ri-plasmid) [18, 37]. However, *A. tumefaciens* is the most preferred strain by researchers for stable expression of the desired protein. In the Ti-plasmid, there are genes encoding for plant hormones such as auxin and cytokinin synthesis, which will induce tumour tissue in plants. However, for vaccine production, these genes will be deleted to form disarmed Ti-plasmid and heterologous gene is inserted forming a recombinant plasmid vector [37]. The recombinant plasmid vector is transformed into *A. tumefaciens* and with the help from *vir* gene of the bacterium, the introduced heterologous gene is transferred by the transformed bacterium and integrated into the host plant nuclear genomic DNA by

nonhomologous recombination at random sites [17, 19, 37]. The transformed bacteria are transferred into the plant leaves by soaking the leaves in the *A. tumefaciens* culture. This method is able to yield a stable integration of the transgene into the genome of the plant [33, 38].

Much research conducted prior to 2000 and early 2000 produced various vaccines through *Agrobacterium*-mediated transformation system in dicotyledonous plant models. For instance, Arakawa et al. successfully expressed gene encoding for cholera toxin B subunit protein in potato leaf explants using *A. tumefaciens* [39]. Similarly, potato has been transformed to produce VP60 protein against rabbit hemorrhagic disease virus, in which rabbits immunized with the potato's leaf extract showed increased anti-VP60 antibody titers and were protected against the hemorrhagic disease [40]. These discoveries have led to the production of more diverse antigens in various crop species. *Helicobacter pylori* TonB protein was expressed in transgenic *Arabidopsis thaliana* through this method [41]. The antigen produced was recognizable by rabbit anti-TonB antiserum and suitable to be used as vaccine against *Helicobacter* infections by oral administration. In addition, Li et al. showed that hepatitis B surface antigen gene was able to be introduced into tomato plants mediated by *A. tumefaciens* [42]. *Agrobacterium*-mediated transformation has also been used for the production of antigens such as heat-labile enterotoxin from *Escherichia coli*, Norwalk virus capsid protein, hepatitis B surface antigen, and transgenic alfalfa expressing proteins from the foot and mouth disease virus by using potato model [43].

Most of the preliminary *Agrobacterium*-mediated transformation studies have been focussing on dicotyledon plants. However, based on recent reports, this method is not limited to dicotyledon plants alone. Yoshida et al. have successfully introduced the Aβ42 gene into rice using the *Agrobacterium* method [44]. When this transgenic brown rice expressing Aβ was orally administered to mice, their serum anti-Aβ antibody titers was elevated [44]. In addition, similar levels of CTB-specific systemic IgG and mucosal IgA antibodies with toxin-neutralizing activity were induced in mice and macaques orally immunized with rice-based oral cholera toxin B subunit vaccine, MucoRice-CTB/Q, or MucoRice-CTB/N produced through *Agrobacterium*-mediated method [45].

Generally, this method allows a large fragment of foreign DNA to be inserted into the Ti-plasmid of *A. tumefaciens*. It is also a simple and cost-effective gene transfer method with higher efficiencies [20, 22]. On the contrary, this method is considered as a rather slow process and produced a low yield [20]. The yield of desired protein is low due to the limitation during the transferring of transformed bacterium into the plant tissues. The soaking method will create the "position effect," in which only the cell layers located at the edge of the explants will receive the transgene and not the whole cells of the explants [16]. Hence, researchers have been refining this expression system by incorporating various possible approaches to improve the production of plant-based vaccines. Among them is incorporation of two agroinfiltration approaches: syringe agroinfiltration as well as vacuum agroinfiltration in the *Agrobacterium*-mediated transformation system.

In an effort to overcome the problems related to the stable expression of the transgene, researchers also use agroinfiltration method to insert the desired gene into plants cells. Agroinfiltration is a method that involves the infiltration of *A. tumefaciens* suspension into the intracellular spaces of desired parts of the plants by using a syringe and results in transient expression of desired protein or transgene [46, 47]. It can improve the expression level of antigen protein in the plant cells [48]. There are two methods of agroinfiltration, which are syringe infiltration and vacuum infiltration. Syringe infiltration is the simplest method where, by using needleless syringe, the transformed *A. tumefaciens* is injected into the leaf. However, creation of a small nick by using a needle has to be done before that and researcher has to ensure that it does not pierce through both sides of the leaf. The advantages offered by this method are multiple transgene constructs which might be introduced into different parts of a single leaf and it can be utilized for a variety of recombinant protein production applications [16]. Despite that, this method allows a rapid and cost-effective analysis on transgene expression level as the procedures involved are relatively simple and do not require sophisticated instruments [16, 49, 50]. On top of that, agroinfiltration can be applied to many plant species after the protocols have been optimised [50].

Another method of agroinfiltration is vacuum infiltration which involves the submerging of the leaves in the infiltration buffer containing transgene-carrying *A. tumefaciens*. A negative atmospheric pressure is subjected to the submerged leaves in the vacuum chamber with the aim of withdrawing the air present in the interstitial spaces of the leaves and occupying the space with the transformed *A. tumefaciens* [51]. Compared to syringe infiltration, this method is more complicated as there is a need for vacuum equipment and reducing the flexibility in the introduction of multiple transgenes on a single leaf. However, it benefits the recombinant protein production through the scalable production process as well as the robustness and rapidness of this method to infiltrate a large number of plants with *Agrobacterium* [51]. *Agrobacterium tumefaciens* has also been utilized to deliver the "deconstructed" viral vectors to lettuce cells and agroinfiltration with geminiviral replicon vectors was found to be able to produce high level of virus-like-particles (VLPs) derived from the Norwalk virus capsid protein (NVCP) and therapeutic mAbs against Ebola (EBV) or West Nile (WNV) viruses in lettuce [51]. Chen et al. further concluded that lettuce is an excellent host for agroinfiltration with deconstructed viral vectors [51].

Table 1 summarises some of the host plants as well as transformation methods used to produce the plant-based vaccines for human and animal diseases.

2.2.2. Genetically Engineered Plant Virus. In this method, a suitable plant virus is modified in order to create chimeric gene for viral coat protein. Thus, it acts as a vector to deliver genetic materials into the plant cells [6, 38]. This method results in transient expression of antigen in plants [6, 38]. The recombinant virus will express the desired protein or peptide

TABLE 1: The plant-based vaccines production for human and animal diseases.

Diseases	Pathogens	Plants	Transformation method	References
Diarrheal	Norwalk virus	*Nicotiana benthamiana*	*Agrobacterium tumefaciens*	[8]
Diarrheal	ETEC	Corn		[61]
Tuberculosis	*Mycobacterium tuberculosis*	*Arabidopsis thaliana*	*Agrobacterium*	[62]
Avian H5N1 influenza	HA protein of H5N1	*Nicotiana benthamiana*	*Agrobacterium*	[2]
Dengue	Dengue virus type 2 E glycoprotein (EIII)	*Nicotiana tabacum* cv. MD609	*Agrobacterium tumefaciens*	[10]
Rabies	Rabies virus	*Nicotiana benthamiana*, tomato	Agroinfiltration	[63]
Hepatitis B	HBsAg	Tomato	*Agrobacterium tumefaciens*	[42]
Foot and mouth disease	FMDV	*Stylosanthes guianensis* cv. Reyan II		[64]
Gaucher disease	Taliglucerase alfa	Carrot	Stable transformation	[65]
Nerve agents attack	Acetylcholinesterase	Tobacco	PEGylated	[66]
Diabetics	Insulin	Safflower	*Agrobacterium tumefaciens*	[5]
Human immunodeficiency	HIV	Tobacco	Agroinfiltration	[67]
Bluetongue	Bluetongue virus	*Nicotiana benthamiana*	Agroinfiltration	[68]
Ebola	ebola virus	*Nicotiana benthamiana*	Agroinfiltration	[69]

ETEC: enterotoxigenic *Escherichia coli*; HA: hemagglutinin; HBsAg: hepatitis B surface antigen; FMDV: foot and mouth disease virus.

as a by-product of viral replication activity during viral infection in the plants [28, 38]. In addition, the synthesis and accumulation of vaccine epitopes can be achieved by modifying the viral capsid proteins [38]. Several advantages of plant virus mediated infection include a high level of recombinant protein expression within a short period of time after infection, easiness to generate multiple antigen copies on the viral particle's surface, and allowing large-scale viral infections in plants [6, 9, 28, 38]. However, products from the viral replication have to be purified first from the infected plants before being used for vaccination [38]. This production method will also cause the death of the plants after infection. Thus, once the vaccine has been harvested, another plant needs to be infected with the recombinant virus and this reinfection procedure has to be done repeatedly for continuous vaccine production [38].

In the earlier development, plant virus expression system involves mostly the engineered RNA viruses such as tobacco mosaic virus (TMV), potato virus X (PVX), alfalfa mosaic virus (AIMV), cucumber mosaic virus (CMV), and cowpea mosaic virus (CPMV) as expression vector [52]. These viruses are not known to replicate in mammalian cells; hence they act as an excellent alternative replicating vaccine vectors for development of both human and veterinary vaccines. Moreover, most of the expression systems have demonstrated that the vaccine antigens produced are protective against challenge infection. For example, the modified CPMV was used to infect *Vigna unguiculata* in the production of a few antigens including VP2 capsid protein of mink enteritis virus (MEV), VP2 capsid protein of canine parvovirus, *Pseudomonas aeruginosa* outer membrane protein F, and *Staphylococcus aureus* D2 domain of fibronectin-binding protein. Subcutaneous injection of the chimeric CPMV expressing the enteritis virus antigen has been shown to protect minks against the challenge with virulent MEV [53].

TMV has also been used to produce various antigens in plant model. Hybrids of TMV containing epitope from murine hepatitis virus (MHV) were propagated in tobacco plants, followed by purification of the virus particles. Mice immunized with the purified hybrid viruses developed serum IgG and IgA specific for the epitope and TMV coat protein. In the study, immunogen administered and protection against MHV infection were found to have a positive correlation [54]. Foot and mouth disease virus (FMDV) epitopes were also expressed in tobacco by the TMV-based vector and guinea pigs, mice, and swine were used to evaluate the protective effects of the recombinant virus. Most of the animals were protected against the FMDV challenge [55]. *Pseudomonas aeruginosa* outer membrane protein F was expressed in tobacco using TMV as well. The chimeric vaccine produced offered immunoprotection against chronic pulmonary infection by *Pseudomonas aeruginosa* in a mouse model [56]. To date, more and more RNA viruses, which also include papaya mosaic virus (PapMV), bamboo mosaic virus (BaMV), tomato bushy stunt virus (TBSV), plum pox, and *Potyvirus* have been added into the list of expression vectors used in plant-based vaccine production [57].

With the advances in plant virus molecular biology, DNA viruses such as geminiviruses have been further developed as one of the state-of-the-art plant expression systems [58]. geminiviruses have a small, single-stranded DNA genome that replicates in the nucleus of host cells by a rolling circle replication mechanism using a double-stranded DNA intermediate [59]. Geminiviral vectors based on bean yellow dwarf virus have been constructed to express its replication initiator protein (Rep) to produce a vaccine against Staphylococcus enterotoxin B (SEB) [60]. In another example, geminiviral replicon system was used to produce an Ebola immune complex (EIC) in leaves of *Nicotiana benthamiana*. Subcutaneous immunization of BALB/C mice with purified EIC resulted in anti-Ebola virus antibody production at levels comparable to those obtained with Ebola glycoprotein (GP1) virus-like particle [59]. Apart from the two examples above, beet curly top virus (BCTV) and tobacco yellow dwarf virus (TYDV) are the other geminiviruses vectors that have been used in the production of various vaccines and therapeutic proteins such as SEB, Norwalk virus VLP, HBVcAg, WNVE protein Mab, HPV-1L1 protein, HIV-1 type C p24, HAV VP1, and vitronectin [58].

3. Methods to Increase the Efficiency of Gene Delivery

Additional methods can be applied in the production of some vaccines with the aim of improving the efficiency of transgene delivery into the host plant tissues. These are by using chemical stimulant and sonication.

3.1. DNA Uptake by Chemical Stimulation. A well-known chemical stimulant which has been used by researchers to accelerate DNA uptake by plant protoplasts is polyethylene glycol (PEG). PEG works by precipitating ionic macromolecules (in this case the DNA), promoting the uptake of the DNA to the protoplasts by endocytosis as well as allowing the transient expression of desired genes [70]. The efficiency of PEG-mediated gene transformation to enhance DNA uptake by protoplasts is depending on several factors such as the concentration of PEG, the inoculation period, and the amount of plasmids [70–72]. The advantages of this method are as follows: (a) it can be incorporated with other gene delivery methods to enhance their efficiency, (b) it will not cause damage to protoplast, and (c) this method is cost-effective as there is no requirement for expensive technical equipment and not much adaptation is required for different protoplasts [70]. However, it can be very difficult to conduct as it requires expertise to carry out the procedures and the successful transformation is highly depending on the genotype of the protoplasts [73]. Furthermore, PEG can also be toxic to the protoplasts and result in a low survival rate and ceased cell division [71]. Due to the complexity of this method, PEG transformation has not been widely used in plant-based vaccine production. Nevertheless, in generating transplastomic tobacco expressing a 25 kda protein antigen against *Mycobacterium leprae* and *Mycobacterium avium*, Hassan et al. successfully transformed the *mmpI* gene along with an adjuvant lymphotoxin-beta (LTB) into tobacco chloroplast by the Polyethylene glycol (PEG) mediated transformation method [74].

3.2. Sonication. Sonication is a technique that utilizes sound waves to agitate particles in solution and, aiming to mix solution, increase the rate of dissolution and remove dissolved gases from liquid. In plant-transformation, sonication will cause the formation of microwounds on plant tissue and enhance the delivery of naked DNA into the plant protoplast [75]. However, it has been reported that sonication alone is not a preferable method for gene delivery in soybean and kidney beans as it caused more negative effects on the plant cells and resulted in low transformation efficiency [75, 76]. In many studies, the sonication assisted *Agrobacterium*-mediated transformation (SAAT) would be used to induce mechanical disruption and formation of wounds on plant cells by ultrasonic waves [77]. The wounded cells will then allow the penetration of *Agrobacterium* into the deeper part of plant tissues, thus increasing the likelihood of plant cells being infected [76]. Being a very easy method, of low cost, and significant to enhance *Agrobacterium*-mediated gene transfer are the benefits of SAAT method. Apart from successful application of SAAT in the transformation of *Chenopodium rubrum* L. [78] and *Beta vulgaris* L. [79], this approach has also been applied in the production of recombinant *Escherichia coli* wild-type heat-labile holotoxin and *Escherichia coli* mutant LT vaccine adjuvants in *Nicotiana tabacum*, in which the highest systemic LT-B-specific IgG titres were detected in birds [80].

Recently, another approach called the combination of sonication plus vacuum infiltration assisted *Agrobacterium*-mediated transformation had evolved to optimise the efficiency of *Agrobacterium*-mediated gene transfer [76]. This approach has successfully transformed the *Fraxinus pennsylvanica* hypocotyls to harbour binary vector pq35GR containing the neomycin phosphate transferase (*nptII*) and *β*-glucuronidase (GUS) fusion gene and an enhanced green fluorescent protein gene [81]. Nevertheless, the application of this combined approach is still scarce and remains at the developmental stage in plant-based vaccines research.

4. Challenges of Plant-Based Vaccines

Although many plant-based vaccines that have been produced are still in phase 1 clinical trials, some vaccines have proceeded or completed phases II and III trials [82]. These therapeutics were produced in various transgenic plants such as insulin in transgenic safflower (SemBioSys), growth factor in transgenic barley (ORF Genetics), taliglucerase alfa in transgenic carrot (Protalix BioTherapeutics), avian influenza vaccine in transgenic tobacco (Medicago), and Ebola Vaccine in transgenic tobacco (Mapp Biopharmaceutical) [82, 83]. Nevertheless, up till today, there is no plant made vaccine that has been approved to be marketed for human consumption. Thus, it is worthwhile to note that even though the production of plant-based vaccines had been initiated almost two decades since 1989 [77], a few challenges still have to be overcome in order to develop them into highly efficacy vaccines. The issues that need to be addressed could start from the upstream processes to the implementation of the vaccines. Generally, three main challenges are the selection of antigen and plant expression host, consistency of dosage, and manufacturing of vaccines according to Good Manufacturing Practice (GMP) procedures.

4.1. Selection of Antigen and Plant Expression Host. The first issue is the selection of an antigen and the right plant expression host [25, 37]. This stage is very important in developing a vaccine that is able to fulfil all the requirements needed because not all antigens are compatible with the selected host plants [37]. The proper and careful selection will not only help to determine the safeness of the vaccine produced, it can also be used to produce thermal-stable vaccine [37]. Meanwhile, identification of antigen candidate of poorly characterised pathogen with promising characteristics can be done by applying genomics or proteomics approaches [23].

4.2. Consistency of Dosage. The consistency of dosage is another challenge that the researchers have to face as dosage produced may vary within the plants of the same species, from fruit to fruit and from generation to generation due to the size and ripeness of the fruits or plants [18, 37]. The transgenic plants show intrinsic variability in the antigen expression due to the position and pleiotropic effects caused by nonspecific integration of the transgene into the host plant genome [25]. On top of that, it is also quite difficult to evaluate the required dosage for every patient. Levels of innate and adaptive immune responses generated in different individuals may vary based on the types of antigens being exposed in the body. Between two patients with different body weight as well as their age, the dosage of plant-based vaccine required will be different. If this issue is not monitored carefully, an immunological tolerance will be induced when the patient is overdosed while reduction in antibody production will occur when the patient is underdosed [37]. Besides that, gene silencing might be induced due to the accumulation of mRNA in the transgenic plant cells as the growth of the plants is stopped and the fruit formation is reduced while the antigen content is increased [84]. In such case, consumption of plant-based vaccines may induce allergic reaction and few side effects such as toxicity on central nervous system, cytokine-induced sickness, and autoimmune diseases [37].

4.3. Manufacturing of Vaccines according to GMP Procedures. The ultimate goal of plant-based vaccines is to produce stable transgenics vaccines which are safe for consumption while reducing the production cost. Besides all the underlying issues that may affect the efficacy of plant-based vaccines, the regulatory guideline regulates by U.S. Department of Agriculture (USDA) and FDA especially the growth of transgenic plants, production and purification of plant-based vaccines, and all phases of clinical trial until marketable stage shall be strictly implemented [63]. Therefore, the manufacturers shall ensure their responsibility to follow the Good Agricultural Practices (GAP) and Good Manufacturing Practice (GMP) so that the upstream to downstream production of plant-based vaccines is strictly controlled for quality management.

Generally, to produce a plant product that could meet the quality standard, the biomanufacturing facilities must be

well equipped so that complete processing cycles of the plant vaccines could be accomplished. The facilities include equipment for plant and bacterium cultivation, infiltration, plant harvest, and protein purification [85]. Takeyama et al. also summarised a few GMP plants that produce various vaccines such as influenza HA antigen, Norovirus capsid protein subunit vaccine, and rice-based cholera vaccine [85]. Concurrently, Kashima et al. reported that in order to produce a plant vaccine that meets the governmental regulatory requirements, a lot of steps and precautions need to be taken into consideration. During the production of a rice-based oral cholera vaccine, MucoRice-CTB, the biomanufacturing agency successfully established specific techniques to maintain the seed of MucoRice-CTB. The agency further evaluated the seed's propagation and stored seeds were renewed periodically to maintain the good quality. Furthermore, cultivation of the plant using a closed hydroponic system helps to minimise the variations in vaccine production. The rice produced was polished, powdered, and packaged to make the MucoRice-CTB drug substance. Final check on the identity, potency, and safety of MucoRice-CTB product must be conducted and only the products that met the quality requirements will be released [86].

It remains a great challenge to maintain the GMP standard for the product in plant-based vaccine industry. Besides the equipment, facilities, and method used to produce the vaccine, other considerations that have to be taken into account are those stated in the GMP guideline published by WHO [87, 88]. GMP for biological products guideline stated that some particular precautions are necessary for the manufacture, control, and administration of biological products as procedures and processes used in the production usually lead to high variation in the quality of products. Thus, the precautious steps should start from the very beginning of the production processes. However, in-process control is also important during the manufacturing of the biological products. Skillful staff are required to run the production processes and thus the biomanufacturing agency should provide necessary training to the staff. Buildings for the vaccine production must be designed in a way that operations can be carried out smoothly. A special design is required for plant vaccine production, in which the seedlots should be stored separately from other materials. Some other general rules of GMP shall be followed to maintain the quality standard of the vaccine products. These include the facts that standard operating procedures shall be implemented for all manufacturing operations, all products shall be clearly labelled, lot processing and distribution records shall be properly kept, and quality assurance and control shall be in place in monitoring the product quality.

5. Future Prospects

Although there are several challenges in the production and application of plant-based vaccines, the development of a better and widely acceptable plant-based vaccine among researchers still remains intact. Research in plant-based vaccine production currently has focused on the development of methods that can increase the amount of antigen produced in the transgenic plants, thus enhancing a significant immune response. The first strategy that can be applied to increase the amount of antigen in the transgenic plant tissues is by optimizing the bacterial or viral genes coding sequence so that the expression is similar to plant nuclear genes [1]. It is also important to determine the suitable subcellular compartment in the plant cells that can yield optimal quantity and quality of antigen.

Besides that, fusion of genes encoding the antigenic protein to immunomodulatory (mucosal adjuvants) proteins can be carried out more extensively as this approach has been shown to have the potential to increase the immunogenicity on the desired antigen. The examples of mucosal adjuvants are bacterial enterotoxins (B subunit of cholera toxin, CTB), secondary metabolites derived from plant, and mammalian and bacterial immunomodulators [89]. This technique is proven to prevent the diarrhoea disease caused by cholera toxin. Through this method, development of multicomponent vaccines is initiated. It has been demonstrated that the fusion of CTB to an enterotoxin protein of rotavirus and *E. coli* adhesion protein has provided defence towards cholera, rotavirus, and enterotoxigenic *E. coli* through the production of tricomponent subunit vaccine in the transgenic potato [89].

Other than development of methods to increase the immunogenicity of plant-based vaccines, more studies are anticipated in overcoming the problem related to dose variability in the transgenic plants. This is following the discoveries by Rigano and colleagues, who showed that some food-processing techniques (batch-processing and freeze drying) could maintain the normal conformation and native antigenicity of material in transgenic plants, such as tomato, potato, and *Arabidopsis* [25], thus standardizing the concentration of antigens in the plants.

6. Concluding Remarks

Plant-based vaccines are the emerging type of vaccines that have a higher therapeutic value to treat many human and animal diseases. A stable and transient gene expression can be obtained based on the gene delivery methods used. By far, chloroplast transformation via biolistic or particle bombardment gene delivery method has been considered as a very promising alternative for better production of plant-based vaccines. However, the development and improvement of suitable gene delivery methods for efficient and optimum vaccine production shall be continued. There are also some bioethical issues arising from the production of plant-based vaccines such as the risk of transferring allergens from transgenic plants to human and animals. As some of the plant-based vaccines use bacteria and virus as the vectors, the pathogens might be reactivated and infect other organisms that consume them. The benefits and advantages of plant-based vaccines shall be able to overwhelm the challenges faced by this interesting biological product. Thus, it is anticipated that regulatory approval will be granted ultimately to help in the global disease control.

Competing Interests

Acknowledgments

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