Chapter

Plant-based Vaccines: The Future of Preventive Healthcare?

Sinan Meriç, Tamer Gümüş and Alp Ayan

Abstract

Infectious diseases threatened humankind countless times through history, when knowledge on microorganisms was absent and medical capabilities were limited. Pandemics and outbreaks caused death of millions, brought empires to their knees and even wiped some ancient civilizations. In "modern" days, despite of improved medical application, sanitary precautions and effective medicines, infectious diseases are still cause of more than 54% of total mortality in developing countries. Millions of people are protected from the infectious diseases annually as a result of mass immunization campaigns. Nevertheless, novel diseases as COVID-19, MERS-CoV, avian influenza, Ebola, Zika and possible future infections require dynamic vaccine research and investment. Along with all the advantages of vaccines, there are several limitations regarding cost, biosafety/biosecurity, storage, distribution, degradation topics. Plant-based vaccine production for humans and animals has been under serious consideration to overcome some of these limitations. Nowadays, plant biotechnology brought new insight to vaccines research through gene transfer strategies to plants and improvements in amount, isolation and purification and addition of adjuvant for production of recombinant vaccine antigens in plants. Recombinant vaccines can undeniably offer us new standards and legal regulations to be introduced for the development, approval, authorization, licensing, distribution and marketing of such vaccines. The aim of this chapter is to exploit uses, methods and advantages of recombinant DNA technology and novel plant biotechnology applications for plant-based vaccine research in respect to existing infectious diseases.

Keywords: Plant-based vaccine, recombinant protein, virus-like particles, transgenic plant, molecular farming, oral vaccine, chloroplast transformation, transient expression, stable nuclear transformation, COVID-19

1. Introduction

There are many breakthrough events during ongoing human civilization process. All of these individual events contributed the process in different significance. Nevertheless, agricultural development and animal domestication significantly accelerated all the other developments due to the fact that they saved people from daily boundary of finding nutrition and allowed more spare time for socialization and thinking.

Advantages of settled life style increased population in early cities rapidly. Ancient cities as Rome, Athens, Fayum varied in population between a hundred thousand to a million in various eras [1, 2]. Lacking the knowledge of

microorganisms, hygiene and sanitation precautions, underdeveloped sewer systems and living so close to domesticated animals and people resulted to the rise of "civilization pathogens". It is hypothesized that virulent pathogens were present but not persistent due to the limited population in human communities before agricultural development and urbanization. Most of the animals tend to live together in herds. Even though the herd lifestyle is very suitable for the transmission of pathogens, there were limited contact between humans and animals since hunting was the only viable way. Developments in agriculture and animal domestication lifted that barrier and allowed animal diseases to be transmitted to humans more frequently in higher population densities. Major fatal human diseases as measles, tuberculosis, smallpox, influenza, pertussis, and falciparum malaria are linked to early domesticated animals via phylogenetic analysis [3]. Throughout the history of human civilization there are several outbreaks of pandemic diseases which shaped the world socially, economically and politically (**Table 1**).

In present day, vaccines are the vital part of the preventive healthcare globally. Many of the once deadly diseases are not present for decades, since the mass vaccination campaigns were applied worldwide. Based on their production method and protection mechanisms, vaccines are categorized under several classes including

Common Name	Year(s)	Cause	Estimated Death (Million)
Plague of Athens	430 B.C.	*Salmonella enterica*	0.1
Antonine Plague	165–180	Small pox (*Variola major* and *Variola minor*) Measles (*Measles morbillivirus*)	5
Plague of Justanian	541–542	*Yersinia pestis*	30–50
Black Death	1347–1352	*Yersinia pestis*	75–200
New World Smallpox	1520 - NA	*Variola major* and *Variola minor*	25–55
Italian Plague	1629–1631	*Yersinia pestis*	1
Great Plague of London	1665	*Yersinia pestis*	0.075–0.1
Third Plague	1885	*Yersinia pestis*	12
Russian Flu	1889–1890	A/H3N8, A/H2N2, or coronavirus OC43 (uncertain)	1
Yellow Fewer	Late 1800s	RNA virus from *Flavivirus* genus	0.15
Spanish Flu	1918–1920	Influenza strains of A/H1N1	50
Asian Flu	1957–1958	Influenza A virus subtype H2N2	1
Hong Kong Flu	1968–1970	H3N2 strain of the influenza A virus	1
HIV-AIDS	1981-ongoing	*Lentivirus*	35 and counting
SARS	2002–2003	Coronavirus (SARS-CoV-1)	<0.001
Swine Flu	2009	H1N1 influenza virus	0.2
MERS	2015	Coronavirus (MERS-CoV)	<0.001
Ebola	2014–2016	Ebolaviruses	0.011
Covid-19	2019-ongoing	Coronavirus (SARS-CoV-2)	2.52 and counting

Table 1.
Major outbreak throughout the history of human civilizations [4].

live attenuated, inactive (killed whole organism), toxoid, subunit (purified native or recombinant protein, polysaccharide or peptide), virus-like particle, outer membrane particle, protein-polysaccharide conjugate, viral vectored, nucleic acid, bacterial vectored, antigen-presenting cell vaccines [5, 6]. Despite the various new approaches to the vaccine production, most of the vaccines which are applied in immunization programs are either live attenuated, inactive or subunit vaccines. WHO (World Health Organization) vaccine-preventable diseases: monitoring system [7] offers important and comparable data on this topic based on all countries. Even so, there are public concerns over the topics as age and schedule of vaccination, common (injection site pain, redness and swelling, fever, malaise, headache) and rare side effects (anaphylaxis, idiopathic thrombocytopenic purpura, narcolepsy, autism), immunodeficiency or antigenic overload issues. As in all daily life matters, misinformation on these topics and issues in social media and search engines greatly challenges vaccine production methods and public acceptance, although the scientific evidences prove the contrary.

As illustrated in **Figure 1**, one of the greatest challenges in vaccine research is based on logistics and distribution. Along with post-production purification and packaging issues, logistics of traditional vaccines under cold chain conditions in limiting shelf-life durations exhibit certain difficulties [6]. Especially in underdeveloped countries, lack of health infrastructure and required conditions threaten overall process. Moreover, world once again faced the same dilemma in Covid-19 pandemic as 16 of total 256 vaccine candidates passed to phase III trials by February, 2021 [8]. These manufacturers announced their plans to produce 10 billion doses (at least 2 doses are required for immunity in most) until the end of 2021 in their best estimations. Apparent insufficient annual production capacity leads priority groups to be formed for the early products. As in this latest ongoing example, inequity in access to vaccines is always a major issue [9].

Leading Covid-19 vaccine candidates are mainly developed by private/industry association by 72%. Remaining 28% is consisted of academic, public and non-profit organizations. Also, there are bigger multinational vast vaccine manufacturer companies as Janssen, Sanofi, Pfizer and GlaxoSmithKline which may ensure tolerating lack of large scale vaccine manufacturing inexperience and capacities of these relatively smaller organizations [10]. Even though, commercial viability

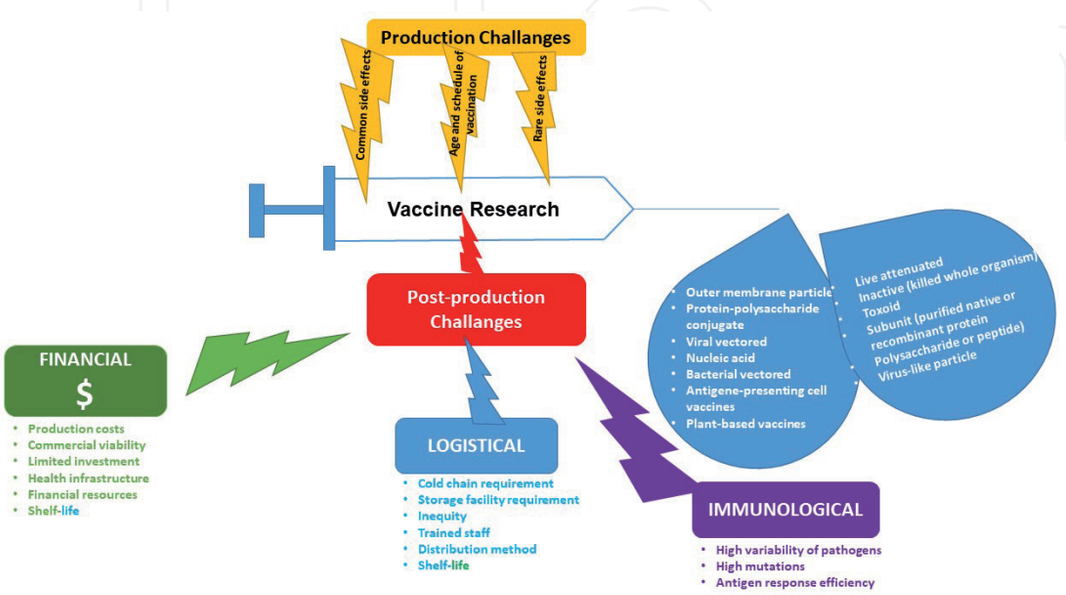

Figure 1.
Main global challenges in vaccine research.

apparently is not an issue for potential Covid-19 vaccine, it is a real drawback for many diseases. These diseases are mostly have devastating effects on restricted local areas as poor communities. In case of such rare infection diseases, development costs offset potential income. Vaccines against this kind of diseases as Ebola, which multinational manufacturers hesitate to invest due to commercial viability, are called 'orphan vaccines' [11]. More profitable vaccine production methods may withdraw hesitation over these diseases which are only producible with government assistance and still have high mortality rates regionally.

Another and probably the most challenging factor in vaccine research is based on immunological issues. Commercially viable vaccine targets for diseases like HIV, gonorrhea (*Neisseria gonorrhoeae*), syphilis (*Treponema pallidum*), malaria or seasonal influenza are generally caused by highly variable pathogens. These pathogens present variation both in and between host variations which emerge difficulty to identify common antigen target for immunization by vaccine. Some people produce natural antibodies against more conserved antigens of pathogens and have enhanced immunity but targeting these conserved antigens by vaccine induction has not been achieved for many of these diseases yet. Protection efficacy is a major issue due to the immunological variations even in post-production of the vaccines [6]. Even so, global reports over novel mutations of the virus will require re-evaluation of efficacy values and raise suspicion in public for existing vaccines. In some case as RTSS vaccine which is licensed for malaria disease, efficacy is lower as 30–40%. Therefore, considering all immunological factors together suggests long development time of traditional vaccines fail to satisfy rapid, flexible and upscale production requirements [6].

Following the rapid development of biotechnology and bioinformatics, precise genomic and proteomic target identification methods and instruments are emerged. Therefore, knowledge on structural biology and immunology is mostly available for many infectious diseases. Deciding vaccine production methods is based on delivery, immunogenicity, production capacity and speed, transport requirements and shelf life and economic viability. Along with the methods as viral vectored vaccines, nucleic acid-based vaccines, RNA vaccines, outer membrane vesicles, plant based vaccines present promising contribution to the field. Experimental and commercial applications of plant based vaccines will be evaluated further in this chapter in perspective of future preventive healthcare alternative.

2. Plant-based vaccine production

Especially in the last two decades, the expression studies of vaccine antigens in plants have been accelerated with the developments in the production of recombinant proteins in plants and it has been provided possibility to design effective plant-based vaccines against many diseases. In this process, both developments in transgenic approaches and transformation methods and improvements in various areas such as promoter selection, codon optimization, plastid transformation for increasing yield of recombinant protein have paved the way for the production of vaccine antigens. The significant increase in the world population and the emergence of epidemic and pandemic diseases cause demands that exceed the vaccine production capacity. However, the success of national vaccine programs is marred by both high cost-per-dose of producing vaccines and the limitations in the distribution of vaccine. In addition, there is a risk of exposure to dangerous pathogens caused by injection procedures during vaccination, resulting in diseases like HIV, hepatite C that can be transmitted through blood. Moreover, risk of contamination of other viruses such as SV40 and foamy virus, which can cause disease in humans and animals, is another important factor that should be evaluated in terms of

health, although it depends on the nature of the vaccine (attenuated or inactivated), the titer of the contaminant, the degree of inactivation and pathogenicity [12]. When all these disadvantages are examined, it is seen that the use of plant systems for vaccine production has the potential to provide a biotechnological solution, considering that it can provide high-scale production and reduce the cost-per-dose and minimize the problems that may occur during vaccine production and distribution. While plants produce complex proteins similar to other eukaryotic systems, they can fold and modify these proteins post-translationally. However, they contain minor differences in glucosylation patterns compared to mammalian cells [13].

In general, the technical points taken into consideration for the plant transformation and the production of recombinant protein in plants should also be taken into account in planning for the production of recombinant vaccine antigens in plant systems. Two different systems are used in the production of recombinant proteins in plants known as stable genetic transformation and transient gene expression. Stable nuclear transformation results in stable expression in plant tissues by ensuring the insertion of recombinant DNA into the nuclear genome of the plant cell [13]. In addition, transgenes can stably integrate into the plastid genome other than the nuclear genome. The transfer of recombinant DNA is carried out by using direct and indirect methods and this preference varies according to the target plant species, target genome (nuclear or plastid), gene construct to be introduced. Natural plant pathogens, *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* are used for indirect gene transfer and are generally preferred for nuclear transformation. Biolistic or microparticle bombardment, which is the most preferred among direct methods, are mostly used in the transformation of the plastid genome and plant species where transformation mediated by *Agrobacterium* species is not applicable. By using plant tissue culture methods with all these transformation methods, a whole transgenic plant can be obtained or plant tissue cultures (callus, hairy root) can be established for recombinant protein production from various plant tissues. In addition, by using plant tissues for recombinant protein production, plant cell cultures also become prominent as an alternative system. Generally, transgenic plants allow large-scale production of recombinant proteins with high expression of introduced gene. This system can also enable the production of multiple recombinant proteins in a single plant by crossing different transgenic plants. On the other hand, development of transgenic plants by stable transformation is relatively more time consuming and also needs improvement due to the low protein expression compared to other plant systems. Another disadvantage is that the transgene may show different profiles in its expression due to positional effect as a result of random entry into the nuclear genome. Moreover, in case of multiple insertions, unstable gene expression and gene silencing may occur. On the other hand, transient expression has many advantages for the expression of genes encoding recombinant proteins in plant tissues. Two processes are particularly prominent for transient transformation in plants named as transient expression of the transgene by *Agrobacterium* infiltration and viral vector-based transient expression. These two processes are based on the expression of transgenes transported by bacteria or virus vectors, and stable integration of the transgene into the genome is not required. The most important advantage of these systems is rapid recombinant protein production. Expression of extra chromosomal transgenes can be detected in 3–4 hours after DNA transfer, while it can reach the maximum expression level in 18–48 hours [14]. Gene expression can be maintained for 10–14 days, afterwards. Transient based expression is generally at a higher level than stable transformation. Plant viral vectors used for viral vector-based transient expression, can be preferred to increase the number of gene copies that can result in a much higher protein yield compared to stable transformation. Various RNA viruses such as tobacco mosaic

virus (TMV), cauliflower mosaic virus (CMV), alfalfa mosaic virus (AVM) are used to construct plant viral expression vectors.

2.1 Nuclear transformation

It is ensured that vaccine antigens can be produced in large amounts in the tissues of transgenic plants that are transformed by nuclear transformation, and oral administration of the vaccine becomes possible thanks to the expression in edible plant organs in such as lettuce (*Lactuca sativa*). The gene integrated into nuclear genome can be maintained with transgenic seeds and replanted when needed. Moreover, by crossing different transgenic plants, different transgenes can be included in a single plant, allowing development of different characteristics of the plant in a short time. Transgenic plant lines, depending on the plant species, can be developed in a shorter time by nuclear transformation than chloroplast transformation. Recombinant vaccine antigens produced by nuclear transformation can be targeted to a variety of organelles such as chloroplasts, vacuoles and endoplasmic reticulum owing to signal peptides, and various post-translational modifications can be achieved particularly in the endoplasmic reticulum. On the other hand, the disadvantage of nuclear transformation is that it displays relatively low levels of expression compared to chloroplast transformation and transient expression, as well as position effect and gene silencing [15].

For many years, the nuclear genome has been the main target of plant gene transfer studies, which has enabled the production of recombinant vaccine antigens by nuclear transformation in plants to come to the fore and to be performed relatively easily. Thanks to nuclear transformation in plants, the production of vaccines against many disease factors from enteric bacteria to viruses that threaten human and animal health has been achieved (**Table 2**).

Every day, 200 million people in the world experience health problems due to gastroenteritis. In developing countries, more than 2 million people die annually from such enteric diseases [23]. It has been reported that a multiepitopic protein from the antigens of enterotoxigenic *E. coli*, *S. typhimurium* and *V. parahaemolyticus* bacteria for use against these diseases is expressed in fresh leaf tissue as much as 5.29 μg g^{-1} in tobacco (*Nicotiana tabacum*) plants by *A. tumefaciens*-mediated transformation. Plant-made LTBentero antigen was found to be immunogenic when administered orally or subcutaneously to BALB/c mice, this antigen was also able to induce specific IgG (systemic) and IgA (mucosal) responses against LTB, ST, and LptD epitopes [23]. Moreover, by regulating the quality processes of transgenic plant-based vaccines, it has been ensured that the quality differences in the production steps are minimized. Especially for this purpose, closed hydroponic plant growing systems were established and oral cholera vaccine (MucoRice-CTB) was produced in accordance with legal regulations within the scope of "Good Manufacturing Practices" without the need for purification [24]. Thus, a plant-based vaccine production system, which can be harvested three times a year and is effective in terms of cost and production, has been implemented. Moreover, Needle- and cold chain free rice-based oral vaccine MucoRice-CTB has been reported to show immunogenicity in humans due to microbiota [25].

In recent years, except advanced monocot and dicot transgenic plants, various moss groups in the plant kingdom have started to be preferred for vaccine production. Env-based HIV (Human Immunodeficiency Virus) multi-epitope protein (poly-HIV) produced in transgenic moss lines has been reported to elicit an immune response in mice as a candidate for subunit vaccine. Moss can be propagated under *in vitro* conditions in accordance with "Good Manufacturing Practices" standards and algae biomass does not have an apparent toxic effect. Therefore, this production system allows immunization with raw moss material. Moreover, mosses

Plant	Pathogen/ Disease	Antigent	Transfer Method	% of Total Soluble Protein or μg of antigen/g fresh biomass	Adjuvant	Immunization	Target Organism	Immun Response	Ref.
Lactuca sativa	Hepatitis B virus	small surface antigen (S-HBsAg)	CaMV 35S promoter/ *Agrobacterium tumefaciens*-mediated	22–63 μg/g FW	With adjuvant (alhydrogel)	Orally-tablet, intramuscularly /total 200 ng	Human	IgA	[16]
Physcomitrella patens	HIV / AIDS	Gp 120 and Gp 41 attached poly-HIV	PEG-Mediated by Protoplast	3.7 μg/g FW	With adjuvant (Freund's complete adjuvant and Freund's incomplete adjuvant)	Subcutaneously/ 34 ng	Human	IgG İnduction	[17]
Nicotiana tabacum	*Mycobacterium tuberculosis*	TBAg-ELP Fusion Protein	CaMV 35S promoter/ *Agrobacterium tumefaciens*-mediated	4% TSB	With adjuvant (Freund's complete adjuvant and Freund's incomplete adjuvant)	Intraperitoneal/ for mice 10 *μ*g, for piglet 360 *μ*g	Human	Induced IgG	[18]
Lactuca sativa	Measles virus	Measles virus hemagglutinin (MV-H)	*Agrobacterium tumefaciens*-mediated	NA	Only intranasal with adjuvant (crude saponins)	Intranasally (40-100 μg-tsb)/ Intraperitonealy/ (400-1000 μg-tsb)/ intramuscularly (50 μg-single dose)	Human (Child)	Induced IgG	[19]

Plant	Pathogen/ Disease	Antigent	Transfer Method	% of Total Soluble Protein or μg of antigen/g fresh biomass	Adjuvant	Immunization	Target Organism	Immun Response	Ref.
Solanum tuberrosum	Infectious bronchitis virus (IBV)	Spike (S) protein	CaMV 35S promoter/ *Agrobacterium tumefaciens*-mediated	2.5 μg/g FTW	Witout adjuvant	Orally 35 g/ intramuscularly 35 g extract	Poultry	Induced chIL-2	[20]
Solanum tuberosum/ *Lycopersicon esculentum*	Norwalk virus (NV)	Recombinant capsid protein rNV	Dual-enhancer 35S promoter /*Agrobacterium tumefaciens*-mediated	ST:120 μg/g FW LE:150 μg/g FW	Without adjuvant	Orally/ 5 g dry weight	Human	Induced serum IgG and intestinal IgA	[21]
Zea mays	Rabies virus RV	G protein	Maize ubiquitin promoter / transformed by biolistics	25 μg/g FW	Without adjuvant	Orally / 0.5–2 mg x 5 fresh kernels	Human and Dog	NA	[22]
Nicotiana tabacum	*E. coli*, *S. typhimurium*, *V. parahaemolyticus*	The LTBentero protein	CaMV 35S promoter/ *Agrobacterium tumefaciens*-mediated	5.29 μg/g FW	With complete Freund's adjuvant / incomplete Freund's adjuvant	Subcutaneously (10 mg)	Human	Induced IgG and mucosal IgA	[23]

FW: Fresh Weight, Tsb: Total Soluble Protein, NA: Not Available, FTW: Fresh Tuber Weight, CaMV: Cauliflower Mosaic Virus, AIDS: Acquired Immune Deficiency Syndrome. ST: Solanum tuberosum, LE: Lycopersicon esculentum.

Table 2.
Plant-based vaccines developed by nuclear transformation/stable expression system.

like *Physcomitrella patens* is able to perform post-translational modifications like N-glucosylation identical to higher plants [17].

Considerable efforts have been made to develop methods that can simplify the purification procedure as the downstream processing of recombinant vaccine antigens will significantly increase production costs. ELPylation as one of these methods, generally increases the accumulation of transgenic proteins in plants as reported in various examples [18]. In addition, ELPylated proteins can be rapidly purified by membrane-based inverse transition cycling (mITC) procedure. Using ELPylation, in which an elastin-like polypeptide (ELP) consisting of a series of pentapeptides is fused to the end of the target protein, an increase in antigen accumulation is observed in both transiently and stably transformed plants [26]. It has also been reported that the immune response increases significantly with the proper folding and trimerization of the antigen. In the study, in which the hemagglutinin of the avian influenza virus (AIV HA) was produced as a monomer and ELPylated trimer form in the tobacco plant (transient in *Nicotiana benthamiana* and stable in *Nicotiana tabacum*), it was observed that the trimeric AIV HA form increased the specific immune response to HA compared to the monomeric form [26].

Virus-like particles that do not contain viral nucleic acids and are formed by viral capsid proteins become prominent as much more reliable candidates when compared with attenuated viral vaccines. Studies in which capsid proteins are expressed in plants by nuclear transformation have been shown to induce specific antibodies comparable to attenuated vaccines when administered orally with an adjuvant [27]. VP2, VP6 and VP7 capsid proteins of group A rotavirus, one of the most common cause of human pathogen, acute infantile and pediatric gastroenteritis worldwide, have been expressed in transgenic tobacco plants. It has been reported to be significantly higher IgG antibodies in the serum of mice of the group VP 2/6/7 (immunized with transgenic tobacco plants) than group VP 2/6 (immunized with transgenic tobacco plants) and the group RV (immunized with orally attenuated RV vaccine) group IgG antibodies are significant. In addition, it was reported that the serum IgG titer in the VP 2/6 group was almost as high as that found in the RV group [27].

Using the nuclear transformation system, plant-based vaccines can be developed on the basis of low-cost multiepitopic recombinant proteins to contain epitope variants that can induce broad-spectrum antibodies. In addition, sequences containing adjuvant activity can be added to these multiepitopic proteins. Multiepitopic vaccines produced in plants trigger local and systemic immune responses more than their counterparts produced in bacteria. In orally inoculated BALBc mice, lettuce-derived HIV C4 (V3) 6 multiepitopic protein showed a higher immunogenic potential than *E. coli*-derived HIV C4 (V3) 6 multiepitopic protein [28].

Plant systems are also used in the production of vaccines against various parasitic diseases other than bacteria and viruses. *Taenia solium* cysticercosis is one of the important parasitic diseases affecting human health especially in developing countries. Production of a low-cost and multi-epitope vaccine against this disease was achieved in the tobacco (*Nicotiana tabacum*) plants by *Agrobacterium*-mediated transformation. The developed transgenic lines were self-pollinated and T1 generation transgenic plants were obtained from the harvested seeds. In these plants, several vaccine-related antigens were expressed in a polyprotein system based on the ribosomal skip mechanism via the 2A sequence derived from the foot-and mouth virus, which induces self-cleavage events at the translational level [29].

2.2 Chloroplast transformation

This section emphasized on recent advances in creation and development of transplastomic plants to produce plant-derived vaccines and protection against

infectious diseases. Plastid transformation has come to be advantageous for the production of vaccines due to the high copy number in a single transformation allowing high levels of transgene expression and the absence of effects leading to gene silencing and the lack of concerns about positional and pleiotropic effects. In addition, as a result of maternal inheritance, the containment of foreign genes in the chloroplast genome and the absence of transgenes in the pollen are other advantages. Moreover, chloroplast transformation ensures expression of multiple genes in prokaryotic-like operon systems. However, this system is limited by the insufficient number of the target plant variety and the trials of very few plant varieties according to nuclear transformation. Another disadvantage is the lack of glucosylation ability of chloroplasts. Therefore, difficulty of expression of eukaryotic human or viral genes in prokaryotic chloroplasts is most important barrier to the use of transplastomic plants. Some major costs associated with the production of recombinant proteins in fermentation-based systems can be reduced by using chloroplasts as bioreactors. Chloroplast-derived therapeutics, especially when administered orally, eliminate expensive purification steps, cold storage, transportation and sterile injection requirements [30].

Advances in transgenic approaches and the development of gene gun and biolistic (particle bombardment method) technologies have enabled the transgene to be transferred directly to living cells. With these systems where tungsten and gold particles are used as microcarriers, the transformation of plastids can be carried out effectively. As an alternative to this system, plastids are transformed via polyethylene glycol (PEG) [31, 32]. PEG-mediated transformation allows the simultaneous transformation of many samples as a simple and efficient method and enables a large number of transformed cells with a high survival rate. However, it has a lower success rate than biolistic-mediated transformation. Despite its high transformation efficiency, biolistic is not available in many laboratories and standardization of its protocol is very difficult. It has been shown repeatedly that recombinant proteins capable of eliciting protective mammalian immune responses can also be produced in chloroplasts of plants. Thanks to chloroplast transformation, vaccines can be produced against viral diseases such as polio (poliovirus), human immunodeficiency (HIV), human papilloma virus (HPV), as well as numerous contagious and fatal bacterial infections and diseases such as cholera, tuberculosis, plague and anthrax (**Table 3**).

Virus-like particles formed by viral capsid proteins that do not contain viral nucleic acids are frequently preferred within vaccine production in plants due to their ability to elicit protective immune responses. In this context, Lenzi et al. [56] reported that the self-assembled L1 capsid protein of human papilloma virus (HPV), that is the causative agent of cervical cancer which is one of the most common causes of death for women, can be produced in chloroplasts of the *Nicotiana tabacum* plant. Thus, in tobacco chloroplasts, the HPV-16 L1 vaccine could be produced by expression of the L1 protein from a natural viral ($L1^{v}$ gene) or a synthetic sequence ($L1^{pt}$ gene) optimized for expression, under the control of plastid expression signals. In addition, accumulation of L1 antigen was obtained only when the first 14 amino acids of the N-terminal domain of the ATPase beta subunit or the Rubisco large subunit were translationally fused to the N-terminal of the L1 protein.

The level of transgene expression in chloroplasts varies depending on the origin of the coding sequence. Although the amount of transcript depends on the high copy number of the transgenes, it is also closely related to the efficiency of the promoter chosen and the regulatory sequences that affect translation. Most of the transgenes expressed in chloroplasts utilize the psbA promoter. It has also been reported that the 5'UTR sequence of psbA shows higher translation activity compared to many 5'UTR sequences. For this reason, studies connected with improving codon optimization benefited from psbA sequences, and it has been reported that

Antigens	Disease/Pathogen	% of Total Soluble Protein or μg of antigen/g fresh biomass	Plant	Immunization	Transfer Method	Ref.
Viral protein 1	Poliovirus	—	*Nicotiana tabacum*	Orally (ORV)	Biolistic	[33]
gp120 and gp41 multiepitope protein	HIV	16 μg /g fresh weight	*Nicotiana tabacum*	Orally (ORV)	Biolistic	[34]
gp120 and gp41 multiepitope protein	HIV	16 μg /g fresh weight	*Nicotiana tabacum*	Orally (ORV)	Biolistic	[15]
ESAT-6 and Mtb72F	*Mycobacterium tuberculosis*	7.5% for ESAT-6 and 1.2% for Mtb72F	*Nicotiana tabacum*	Orally (ORV)	Biolistic	[35]
mmpI with Lymphotoxin-beta (LTB)	*Mycobacterium leprae and Mycobacterium avium*	—	*Nicotiana benthamiana*	—	Polyethylene glycol (PEG)	[36]
C4V3 polypeptide	HIV	—	*Nicotiana tabacum*	Orally (ORV)	Biolistic	[37]
L1 capsid protein fused with LTB	Human papillomavirus	2%	*Nicotiana tabacum*	—	Biolistic	[38]
dengue-3 serotype polyprotein (prM/E)	Dengue virus	—	*Lactuca sativa*	Orally (ORV)	Biolistic	[39]
cholera toxin-B AMA1 MSP1	*Vibrio cholera* (Cholera)-Plasmodium (Malaria)	13.17 and 10.11%	*Nicotiana tabacum*	Subcutaneously (SQV) or orally (ORV)	Biolistic	[40]
cholera toxin-B AMA1 MSP1	*Vibrio cholera* (Cholera)-Plasmodium (Malaria)	7.3 and 6.1%	*Lactuca sativa*	Subcutaneously (SQV) or orally (ORV)	Biolistic	[40]
D2 fibronectin-binding domain+CTB	*Staphylococcus aureus*	0.7%	*Chlamydomonas reinhardtii*	Orally (ORV)	Biolistic	[41]
VP1	Foot and mouth dis ease virus (FMDV)	51%	*Nicotiana tabacum*	Inoculated	Biolistic	[42]
HIV-1 $Pr55^{gag}$ polyprotein	HIV	6.75%	*Nicotiana benthamiana*	—	Biolistic	[43]
labile toxin B subunit heat-stable toxin (LTB-ST)	ETEC-induced diarrhoeal disease	2.3%	*Nicotiana tabacum*	Orally (ORV)	Biolistic	[44]

Antigens	Disease/Pathogen	% of Total Soluble Protein or μg of antigen/g fresh biomass	Plant	Immunization	Transfer Method	Ref.
E7 and E7CP HPV protein	Human papillomavirus	0.1% and 0.5%	*Nicotiana tabacum*	—	Biolistic	[45]
multi-epitope DPT fusion protein	*Corynebacterium diphtheria, Bordetella pertussis, Clostridium tetani*	0.8%	*Nicotiana tabacum*	Orally (ORV)	Biolistic	[46]
F1-V	*Yersinia pestis* (Plague)	14.8%	*Nicotiana tabacum*	Orally (ORV)	Biolistic	[47]
HIV-1 Pr55Gag, p24 and p17/p24	HIV	4 μg/g fresh weight	*N. benthamiana*	Intramuscularly	*A. tumefaciens* (chloroplast-targeted)	[48]
L1 capsid protein	Human papillomavirus	24%	*Nicotiana tabacum*	Intraperitoneal (IP)	Biolistic	[49]
L1 capsid protein	Human papillomavirus	1.5%	*Nicotiana tabacum*	—	Biolistic	[50]
Structural protein E2	Swine fever virus (CSFV)	1–2%	*Nicotiana tabacum*	Subcutaneous and intragastric	Biolistic	[51]
TetC	*Clostridium tetani* (Tetanus)	10%	*Nicotiana tabacum*	Oral and İntranasal (IN)	Biolistic	[52]
SARS-CoV Spike Protein	Severe acute respiratory syndrome coronavirus	0.2%	*Nicotiana tabacum*	Orally (ORV)	Biolistic	[53]
Protective antigen (PA)	*Bacillus anthracis* (Anthrax)	2.7 and 1.7%	*Nicotiana tabacum*	—	Biolistic	[54]
VP6	Rotavirus	3%	*Nicotiana tabacum*	—	Biolistic	[55]

Table 3.
Plant-based vaccines developed by chloroplast transformation.

codon optimization significantly increases translation in chloroplasts [57]. It has been indicated that the expression of the gene of the vaccine subunit is increased 50 times in chloroplasts by codon optimization [33].

In recent years, the production of vaccine antigens in the chloroplasts of edible plants like lettuce and ensuring oral vaccination have come to the fore as an important development in order to eliminate the economically demanding and extremely expensive steps such as fermentation, purification, cold storage, cold chain and transportation. Lyophilized plant cells stored at ambient temperature retain their efficacy and antigen folding/assembly, thus eliminating the need for cold chain [33]. Thus, chloroplast bioreactors have become an important alternative to the production of fermentation-based vaccine antigens. Arlen et al. [47] have achieved the production of high levels of F1-V antigen, as much as 14.8% of the total soluble protein. In a study conducted by aerosol challenge with *Y. pestis*, 33% of subcutaneously F1-V (with adjuvant) immunized mice survived, while 88% of orally F1-V immunized mice survived. These data presented that oral doses of vaccine antigens produced in chloroplasts may be effective in eliciting protective immune responses in vivo. In yet another study, an increase in IgG1 and IgA titers was reported with oral boosting of low-cost, cold chain-free, chloroplast made viral protein 1 (VP1) subunit vaccine [28]. By producing multiple vaccine antigens in chloroplasts, immunity can be improved against more than one infectious disease at the same time. Cholera toxin-B subunit (CTB) fused malarial vaccine antigens apical membrane antigen-1 (AMA1) and merozoite surface protein-1 (MSP1), expressed in lettuce and tobacco chloroplasts inhibited the proliferation of malaria in red blood cells by inducing antigen-specific antibodies in mice [40].

Besides terrestrial plant systems, photosynthetic unicellular alga *Chlamydomonas reinhardtii*'s chloroplasts are used for vaccine production against various pathogens. Algae vaccines can remain stable at room temperature for more than 1.5 years and show faster and more controllable growth characteristics than other members of the plant kingdom. D2 fibronectin-binding domain of CTB fused *Staphylococcus aureus* was stably expressed in *C. reinhardtii* algae and as a result of algae-based inoculation of mice, the amount of pathogen decreased and 80% of mice were protected against the lethal dose of *S. aureus* [41]. It was reported in another study that the fusion protein was developed against tuberculosis caused by *Mycobacterium tuberculosis*, one of the leading fatal diseases, can be stored in lyophilized leaf for up to 6 months at room temperature and preserves its stability and proper folding/assembly [36].

Production of vaccine antigens (HIV gag transgene; $Pr55^{gag}$) by biolistic-mediated chloroplast transformation resulted in significantly greater protein accumulation than *Agrobacterium*-mediated nuclear transformation which vaccine antigens postranslationally targeted into plastids [43]. In transient expression experiments, various cellular organelles (cytosol, apoplast, endoplasmic reticulum, chloroplast and mitochondrion) were targeted. It was reported that specific $Pr55^{gag}$ sequences were expressed only in chloroplasts. The synthetic gene encoding the HIV C4V3 recombinant protein known to induce both systemic and mucosal immune responses in mammalian systems was expressed in chloroplasts of *Nicotiana tabacum* plants. It has been reported that the obtained plant-derived C4V3 elicits systemic and mucosal antibody responses in BALB /c mice by oral immunization [37]. Multepitopic protein (Multi-HIV) derived from HIV gp120 and gp41 envelope proteins expressed in tobacco chloroplasts also induced immune responses and T-helper specific responses by oral immunization in BALB/c mice [34].

2.3 Plant virus based expression system

Plant viruses are generally described as safe for humans and animals. Therefore, they are preferred for the production of therapeutic molecules. TMV (tobacco

mosaic virus), PVX (potato virus X), BaMV (bamboo mosaic virus), CPMV (cowpea mosaic virus) are highly stable to high temperature, pressure and pH conditions and can be purified from host plants in amounts exceeding hundreds of mg/kg plant biomass [58]. Virus-like particles (VLPs) are multi-subunit molecules consist of self-assembly protein structures that are the same or highly similar to the general structure of authentic virus. Due to the fact that VLPs do not contain viral nucleic acids, therefore conversion to infectious viruses is not possible which is an important risk factor for attenuated vaccines. In recent years, the use of both VLPs and plant viruses for vaccine production has increased rapidly (**Table 4**). There are various recombinant vaccine production strategies that stand out in which different vaccine antigens can be produced by using plant viral structures. Launch vector-based on virus for plant transient expression system, plant virus-based vector systems by the incorporation of 2A peptide, plant virus conjugated with purified antigen from bacterial expression system are some of the systems by which recombinant vaccine antigens can be produced.

Multiple doses of multivalent vaccine can be administered in the immunization of mice without any adverse effects. In addition, multivalent subunit vaccines can be developed against various diseases using an efficient TMV-based delivery platform. A multivalent subunit vaccine consisting of the combination of OmpA, DnaK chaperone and Tul4 protective antigens of the *Francisella tularensis* pathogen bacterium has been reported to be safe. *F. tularensis* proteins were chemically conjugated to the TMV surface and the developed subunit vaccine strongly induced humoral immune response [60]. Chen et al. [59] produced a candidate vaccine (BJ2A CVP) in *Chenopodium quinoa* and *N. benthamiana* against Japanese encephalitis virus (JEV) using a bamboo mosaic virus-based chimeric virus particle (CVP) strategy. *Chenopodium quinoa* plant is preferred to reduce the side effects of nicotine and other alkaloids found in tobacco species in the purification of BJ2A CVP.

The genomes of both RNA and DNA viruses can be modified for recombinant protein production. Geminiviral replicon systems are one of the plant-viral based expression systems used to increase the expression of vaccine antigens in plants. In geminiviral derived vectors with a single stranded DNA genome, the viral genes encoding the coat and movement proteins are deleted and the expression cassette for protein of interest is inserted. In these strategies, it has been reported that the viral vector has transient expression only 4 days after it was transferred to *Nicotiana benthamiana* plant leaves by *Agrobacterium* infiltration and maintained this expression level up to 7 days [70]. On the other hand, by using plants with low secondary metabolite content such as lettuce in geminiviral replicon systems based on bean yellow dwarf virus, virus-like particles could be produced at high expression levels. Thus, vaccine candidates such as Norwalk virus capsid protein-VLP (NVCP-VLP) can be purified from lettuce plants without losing their functional activity. Plant virus-based expression methods along with *Agrobacterium*-mediated agroinfiltration are often preferred for increasing low expressions of vaccine antigens in plants and improving the feasibility of plant-based vaccines. The expression of recombinant proteins were carried out in *Nicotiana benthamiana* plant by using the Launch vector-based plant transient expression system with agroinfiltration, and it was reported that vaccine candidates caused up to 100% protection against diseases that could be used for bioterrorism such as anthrax after purification [63]. In another study, it has been reported that Consensus domain III of dengue virus E glycoprotein (cEDIII) shows high expression with plant virus-based expression (5.2 mg/g dry weight of leaf tissues) against Dengue virus [61]. Launch vector technologies, which are used in the production of VLP-based recombinant vaccines especially against infectious diseases, are also used in the production of protective vaccines such as malaria transmission blocking vaccines (TBVs). TBVs prevent successful

Antigens	Disease/ Pathogen	Recombinant vaccine platforms for production of antigen	Plant	Immunization	Virus	Ref.
JEV envelope protein domain III (EDIII)	Japanese encephalitis virus (JEV)	BaMV-based vector system	*Chenopodium quinoa*	Intraperitoneally (IP)	Bamboo mosaic virus (BaMV)	[59]
OmpA-like protein (OmpA), chaperone protein DnaK and lipoprotein Tul4	*Francisella tularensisis* (Tularemia)	Bacterial system (*E. coli*) and chemically conjugated	*Nicotiana benthamiana* for TMV-Lysine production)	Intranasally (IN), subcutaneously (SC.)	Tobacco mosaic virus (TMV)	[60]
Dengue virus envelope glycoprotein (E) domain III	Dengue virus	TMV-based vector system	*Nicotiana benthamiana* (Agroinfiltration)	Orally (ORV)	Tobacco Mosaic Virus (TMV)	[61]
RhoA-derived peptide (Antiviral peptide production/inhibitor)	Respiratory syncytial virus (RSV)	TMV-based vector system	*Nicotiana benthamiana* (Agroinfiltration)	—	Tobacco Mosaic Virus (TMV)	[62]
Protective antigen (PA)	*Bacillus anthracis*	Launch vector-based plant transient expression system	*Nicotiana benthamiana* (Agroinfiltration)	Intramuscularly (IM)	Tobacco mosaic virus (TMV)	[63]
Pfs25 VLP	*Plasmodium falciparum* (Malaria)	Launch vector-based on Tobacco mosaic virus	*Nicotiana benthamiana*	Intramuscularly (IM)	Tobacco Mosaic Virus (TMV)	[64]
Norwalk virus capsid Protein (NVCP)	Norwalk virus	BeYDV-based geminiviral replicon system	*Lactuca sativa* (Agroinfiltration)	—	Bean yellow dwarf virus (BeYDV)	[65]
PCV2 capsid protein	Porcine Circovirus (PCV)	CMV-based expression system	*Nicotiana tabacum* (inoculated with purified virions)	Intraperitoneally (IP)	Cucumber mosaic virus (CMV)	[66]
Extracellular domain M2 protein (M2e) fused to hepatitis B core antigen (HBc)	Influenza	Potato X virus-based vector system	*Nicotiana benthamiana* (Agroinfiltration)	Intraperitoneally (IM)	Potato X virus (PXV)	[67]

Antigens	Disease/ Pathogen	Recombinant vaccine platforms for production of antigen	Plant	Immunization	Virus	Ref.
Hemagglutinin (HA) protein	H5N1 influenza virus	Launch vector-based plant transient expression system	*Nicotiana benthamiana* (Agroinfiltration)	Subcutaneously (s.c.)	Tobacco mosaic virus (TMV)	[68]
BTV coat proteins	Bluetongue virus (BTV)	Cowpea mosaic virus–based HyperTrans (CPMV-HT) and associated pEAQ plant transient expression vector system	*Nicotiana benthamiana*	Subcutaneously (s.c.)	Cowpea mosaic virus	[69]

Table 4.
Plant-based vaccines developed by plant virus-based expression system.

sporogonic development of the sexual stage *Plasmodium falciparum* parasites ingested by female *Anopheles* mosquitoes [64]. Thus, the spread of parasites in endemic populations by transmission from human to mosquito is prevented [41]. Another plant virus-based expression approach is the fusion of the vaccine antigen to the virus-like carrier particle, either genetically or by chemical cross-linking [67]. It has been reported that the potato X virus-based recombinant viral vector provides a high level of expression of the hybrid protein consisting of influenza virus M2 protein (M2e) fused to hepatitis B core antigen (HBc) in *Nicotiana benthamiana* plants. This vector was transferred to plant leaves by agroinfiltration and the hybrid protein was synthesized to 1–2% of the total soluble protein.

2.4 Transient expression

In addition to stable transgene expression (nuclear or plastid transformation), transient expression is often preferred for the expression of genes encoding vaccine proteins in plant tissues. Since transient expression does not contain chromosomal integration, it is not affected by position effect. In addition, the expression of extrachromosomal transgenes can be detected 3 hours after transfer and can last for about 10 days [14]. The major advantage of transient expression is production of vaccine antigen rapidly at low cost and high yield. In addition, the easy applicability of the system and its ability to allow the production of complex proteins composed of subunits encourages its use against the novel viral diseases that emerge suddenly. Plant-based vaccines developed by transient expression are given in **Table 5**.

Margolin [73] achieved *Agrobacterium*-mediated transient expression of soluble HIV Env gb140 antigens in *Nicotiana benthamiana* plant. It has been reported that rabbits immunized intramuscularly with lectin affinity purified antigens developed binding antibodies and neutralizing antibodies at high titers. The production of nucleo capsid (N) and membrane protein (M), two important antigens of SARS-CoV, was achieved in *Nicotiana benthamiana* plant with transient expression created by using both virus-based vector and agroinfiltration. In addition, tobacco leaves infiltrated with *Agrobacterium tumafaciens* C58 and GV3101 strains harboring the pBI-Mvector containing the M protein, SARS-CoV M protein production was successfully achieved without using any post-transcriptional gene silencing suppressors [78].

It is imperative to produce vaccines at rates which offset mutation frequency of viral infections such as influenza, for which a new and unique epidemic strain appears within a few years. In recent years, recombinant vaccines become prominent as one of the most important options to solve this problem. New recombinant strategies provided by plant biotechnology and the production of plant-based vaccines are becoming widespread in the struggle with pandemic and epidemic diseases such as Influenza A H1N1, Influenza H5N1, plague, Ebola, Zika, SARS-CoV and SARS-Cov-2 [78–81]. Plant-based vaccines developed for pandemic and epidemic diseases are given in **Table 6**.

Especially the commercial scale production of these vaccines and their examination at Phase I, Phase II and Phase III levels beyond the functional evaluations in animal models indicates that in the future, plant-derived vaccines will be an important part of the struggle against pandemic and epidemic diseases. Plant-based vaccines produced in commercial scale and candidate vaccines are given in **Table 7** by companies. For instance, COVID-19 (severe acute respiratory syndrome coronavirus 2/SARS-CoV-2), which has become a major threat to global health, has also significantly impacted the world economy and social mobility. So far, with its high contagiousness, rapid spreading nature and high mortality rate, more than 2.5 million people have died from COVID-19, and more than 116 million people have

Plant	Pathogen/ Disease	Antigent	Transfer Method	% of Total Soluble Protein or μg of antigen/g fresh biomass	Adjuvant	Immunization	Target organism	Immun response	Ref.
Nicotiana benthamiana	HPV-16/ Cervical cancer	E7 protein fused 16E7SH	*Agrobacterium tumefaciens*-mediated	0.4–6 g/kg FW	With adjuvant/ (Freund's incomplete adjuvant)	Subcutaneous/ 5 μg	Human	Tumor size decrease and IgG ınduction	[71]
Nicotiana benthamiana	Hepatitis B virus	HBsAg	MagnICON viral Vectors/ *Agrobacterium tumefaciens*-mediated	0,64 mg/g FW	With adjuvant (alum)	Intraperitoneal 346 mIU/mL at week	Human	anti-HBsAg response	[72]
Nicotiana benthamiana	HIV/AIDS	Subtype C Envelope gp140	*Agrobacterium tumefaciens*-mediated	4.9–6.2 mg/kg FW	With adjuvant (Alhydrogel®)	Intramuscularly/ 50 μg	Human	NA	[73]
Nicotiana benthamiana	Poliovirus (PV) type 3	Capsid protein VP1	*Agrobacterium tumefaciens*-mediated	60 mg/kg FW	—	Intraperitoneal / intramuscular	Human	—	[74]
Nicotiana benthamiana	Infectious Bursal Disease Virus (IBDV)	Structural VP2 protein	*Agrobacterium tumefaciens*-mediated	1% TSB	With adjuvant (Freund's complete adjuvant and Freund's incomplete adjuvant)	Intramuscular/ (12 μg of VP2)	Poultry	NA	[75]
Nicotiana benthamiana	FMDV	Capsid precursor P1-2A and the protease 3C fusion	*Agrobacterium tumefaciens*-mediated	3–4 mg/kg FW	With adjuvant (Montanide ISA 50)	Intraperitoneal/ 500 ng	meat-producing animals	NA	[76]

Plant	Pathogen/ Disease	Antigent	Transfer Method	% of Total Soluble Protein or μg of antigen/g fresh biomass	Adjuvant	Immunization	Target organism	Immun response	Ref.
Nicotiana benthamiana	*Plasmodium falciparum*/ Malaria	Plasmodium Surface Protein Pf38 fused to Red floresan Protein	CaMV 35S promoter/ *Agrobacterium tumefaciens*-mediated	4–12 μg/g FW	With Gerbu MM Adjuvant	Intraperitoneal (17 μg)	Human	Induced IgG	[77]

FW: Fresh Weight, Tsb: Total Soluble Protein, NA: Not Available, CaMV: Cauliflower Mosaic Virus.

Table 5.
Plant-based vaccines developed by transient expression system.

Spreading area	Plant	Pathogen/ Disease	Antigent	Transfer Method	% of Total Soluble Protein or μg of antigen/g fresh biomass	Adjuvant	Immunization	Immun response	Ref.
E	*Oryza sativa*	*Vibrio cholerae*/ *Cholera*	Cholera toxin B-subunit	*Agrobacterium tumefaciens* mediated	NA	Witout adjuvant	Orally/150 mg seed	Induced IgG and mucosal IgA	[82]
E	*Nicotiana benthamiana*	SARS-CoV	SARS-CoV nucleocapsid (rN) protein	*Agrobacterium tumefaciens* mediated	0.8–1% of the TSP	With complete Freund's adjuvant/ incomplete Freund's adjuvant	Intraperitoneal/500 mg fresh tobacco leaves	Induced IgG1 and IgG2/ Increased IFN-_ and IL-10/not changed IL-2 and IL-4	[83]
P	*Nicotiana benthamiana*	Influenza A H1N1	Soluble protein H1/ H1-VLP	*Agrobacterium tumefaciens* mediated	NA	-	-	Induced CD4+ and CD8+ T cells	[84]
E	*Nicotiana benthamiana*	Influenza A H5N1	Matrix protein 2 ectodomain (M2e) fused to N-terminal proline-rich domain (Zera®) of the γ-zein protein of maize	*Agrobacterium tumefaciens* mediated	125–205 mg/ kg FW	Without adjuvant	Intramuscular/4.5 μg	Induced IgG	[85]
E	*Nicotiana tabacum*/*Lycopersicon esculentum*	*Yersinia pestis*/Plague	Major capsular protein F1-V antigen fused	*Agrobacterium tumefaciens* mediated	NT: 1-4% FW LE: 4-10% mg DW	With adjuvant NT: aluminum hydroxide t/LE: cholera toxin	NT: Subcutaneously (10μg purified)/LE: Orally (2 g tomato fruit)	Induced serum IgG1, IgG2a and mucosal IgA	[86]

Spreading area	Plant	Pathogen/ Disease	Antigent	Transfer Method	% of Total Soluble Protein or μg of antigen/g fresh biomass	Adjuvant	Immunization	Immun response	Ref.
E	*Nicotiana benthamiana*	Flavivirus/ Yellow fever (YF)	YF virus envelope protein (YFE) fusion to the bacterial enzyme lichenase (YFE-LicKM)	*Agrobacterium tumefaciens* mediated	NA	With Alhydrogel adjuvant	Intramuscularly 5 μg x3/5 μg x 2/30 μg x 3	Induced IgG, İncreased IFNγ	[87]
E	*Nicotiana tabacum*	Ebola virus (EBOV)	Envelope-associated protein VP40	*Agrobacterium tumefaciens* mediated	2.6 μg/g FW	With complete Freund's adjuvant/ incomplete Freund's adjuvant	Orally (25 ng)/ Subcutaneously (125 ng)	Induced IgM, IgG and intestinal IgA	[88]
E	*Nicotiana benthamiana*	Zika virus (ZIKV)	Envelope (E) protein	*Agrobacterium tumefaciens* mediated	160 μg/g FW	With aluminium hydroxide gel adjjuvant	Subcutaneously/50 μg x 24	Induced IgG1 and IgG2, Increased IFN-γ, IL-4 and IL-6	[89]
P	*Nicotiana benthamiana*	SARS-CoV-2	Spike specific monoclonal antibody (mAb) CR3022	*Agrobacterium tumefaciens* mediated	130 μg/g FW	-	-	-	[90]

P: Pandemic, E: Epidemic, FW: Fresh Weight, Tsb: Total Soluble Protein, NA: Not Available, DW: Dry Weight. NT: Nicotiana tabacum, LE: Lycopersicon esculentum.

Table 6.
Plant-based vaccines developed for pandemic and epidemic diseases.

iBio, Inc			[91]
IBIO-201 Prophylaxis of SARS-CoV-2 spike protein fused lichenase protein / *Nicotiana benthamiana*	IBIO-400 Prophylaxis of Classical swine fever (CSF) CSFV E2 glycoprotein / *Nicotiana benthamiana*		
Medicago Inc.			[92]
MT-7529 Prophylaxis of H7N9 influenza / Phase 2 / *Nicotiana benthamiana*	MT-2355 Prophylaxis of pertussis, diphtheria, tetanus, poliomyelitis and prophylaxis of Hib infection in infants / Phase 3 / *Nicotiana benthamiana*	MT-2271 Prophylaxis of seasonal influenza / *Nicotiana benthamiana*	
MT-5625 Prophylaxis of rotavirus gastroenteritis / Phase 1 / *Nicotiana benthamiana*	MT-2766 Prophylaxis of SARS-CoV-2/ Phase 2 / *Nicotiana benthamiana*	MT-8972 Prophylaxis of H5N1 influenza / Phase 2 / *Nicotiana benthamiana*	
Icon Genetics			[93, 94]
ZMapp Prophylaxis of Ebola virus / *Nicotiana benthamiana*	Norovirus Vaccine Prophylaxis of Norovirus / Phase I / *Nicotiana benthamiana*		
SoyMeds, Inc.			[95]
soy-mSEB Prophylaxis of Staphylococcal Enterotoxin B / *Glycine max*			
Fraunhofer CMB			[96]
Prophylaxis of H1N1 influenza	Prophylaxis of H1N1 influenza	Prophylaxis of Malaria	

Table 7.
Plant-based vaccines producing companies and commercial products.

been infected with COVID-19 worldwide [97]. In order to control the pandemic, the whole world work hard to develop new strategies to be applied in the field of health, to deliver vital medical supplies to those in need, to develop and apply safe and effective vaccines. Especially, the development of vaccines and drugs for this new pathogenic Coronavirus, which emerged suddenly and mutated at certain times, became an inevitable target. Until now, the number of vaccines in preclinical development are 182 and the number of vaccines in clinical development is 74, worldwide [8]. Plant-based vaccines have also proven that they can play an active role in fighting against COVID-19 with their promising results in preclinical and clinical stages. Along with the initiative of commercial companies using plant biotechnology, transient expression of the SARS-CoV-2 antigen in plants was achieved, and a plant-based COVID-19 vaccine candidate was produced with a high-scale production technology [81]. The COVID-19 vaccine developed by Medicago company using a plant-based platform started Phase II clinical trials. In this approach, virus-like particles (SARS-CoV-2 spike protein self-assambles into VLPs) could be produced by transient expression in *Nicotiana benthamiana* plants within just 20 days after the acquisition of SARS-CoV-2 [81]. For Phase I, vaccine administration in healthy adults was performed as two intramuscular doses and at 3 different dose levels (S protein content 3.75, 7.5 or 15 μg), either alone or with adjuvant (AS03 or CpG1018). After the second dose administration, the adjuvanted CoVLP was able to induce humoral and cellular responses for all dose levels. Another company,

BAT's US Bio-tech arm, Kentucky BioProcessing (KBP), announced that the COVID-19 vaccine candidate will be evaluated in the Phase I trial after the Food and Drug Administration (FDA) accepts the Investigational New Drug application. This candidate vaccine was developed using plant-based technology and it was stated that the active ingredient of the vaccine could be rapidly produced in a short period of 6 weeks according to conventional methods. In still another initiative, iBio, Inc. company announced that it was working on the subunit IBIO-201 candidate vaccine against SARS-CoV 2 immediately after the pandemic was declared. The candidate vaccine was produced by *Agrobacterium*-mediated infiltration in leaves of *Nicotiana benthamiana* plants and the target protein was purified and formulated for the final product. The candidate vaccine has been reported to induce the production of anti-spike neutralizing antibodies in immunized mice. Due to the new mutations of SARS-CoV-2, it has become important to produce subunits or virus-like particles as vaccine candidates at low cost-per-dose and higher production scale.

2.5 Evaluation of plant-based vaccines side effect

Vital part of vaccine research is the risk assessment through randomized, double-blind placebo-controlled multicentre trials. Vaccine side effects can be evaluated under two categories as common side effects (high fever, vomiting, dizziness, anxiety and nausea) and rare side effects (risk of hospitalization, death or long-term morbidity). Same evaluation processes is required for plant-based vaccines as all traditional and recombinant vaccines. In literature, there are various studies in emphasis to safety and side effects of plant-based vaccines.

Plant-based vaccines can be evaluated in two different ways: cases in which the plant content is directly applied in pure form and the cases where the vaccine content is isolated and mixed with adjuvant. Phase studies were initiated for many candidate vaccines, where antigen or VLP was produced and then mixed with adjuvant before injection. In reported Phase I vaccine case against influenza A presented common side effects on volunteers as high fever, vomiting, dizziness, anxiety and nausea [96]. Similarly, in another case local effects occurred in the vaccination area and 93% of side effects were mild effects [98]. McCormick et al., stated in their studies that volunteers showed symptoms that were described as severe at a very low rate, but recovered within 1–2 days without the need for medical intervention, and the vaccine candidate was quite safe [99]. Moreover, Pillet et al. tested the vaccines with 300 healthy adults and 450 volunteers over the age of 50 in their phase III study on two different age groups. As a result, a higher rate of fatigue was observed in volunteers over 50 years old [100]. Ward et al. reported that the most common side effect was pain at the injection site in their studies on 22,854 volunteers and mortality rate was slightly higher for inactivated.

vaccine comparing to virus like particles [101]. Chichester et al., (2018) reported that 94% of the volunteers developed at least one of the side effects of high fever, vomiting, dizziness, anxiety and nausea [102]. In all of these studies with both oral and injectable vaccines, the observed effects were evaluated as mild to moderate. In this way, plant-based production has been defined as an effective and safe vaccine production tool [103]. Production of the adjuvants to be used to stimulate mucosal and peripheral immunity in the plant or the selection of plant which produce appropriate secondary metabolites that can act as mucosal adjuvants contributes to the decrease in the incidence of side effects. The number of studies in which plant-based vaccine candidates have passed to phase studies were much less than classical vaccine studies. When these studies are evaluated, there is no significantly increased side effect risk report concerning plant-based vaccines against any other vaccines production options [104].

2.6 Legal regulations involving plant-made pharmaceuticals

Regulatory processes of the vaccine development, approval, authorization, licensing, distribution, and marketing are as challenging as the production. There are both national and centralized regulatory agencies. These agencies emphasize on scientific evaluation of data, quality of the product, safety for human use, verification of reported efficacy and authenticity of product labels. In USA, centralized regulatory agency is Food and Drug Administration known as FDA. As plant based-vaccines are considered in biological materials apart from chemical entities, plant-made pharmaceutics are regulated under Biologics License Application (BLA) [105]. The European Union (EU) members have both their own national regulation and the centralized regulation under European Medicines Agency (EMA) which acts as the counterpart of FDA in Europe. The Committee on Herbal Medicinal Products (HMPC) is the EMA's committee responsible for compiling and assessing scientific data on herbal substances, preparations and combinations, to support the harmonization of the European market [106]. However, considering the nature of plant based vaccines, they are under authorization of The Committee for Medical Products for Human Use (CHMP) which plays a vital role in the authorization of medicines in the European Union (EU).

Strictness of the regulation is mostly based on the production method and the host plant. Non-food plants as *Nicotiana* species and the controlled indoor production methods as plant tissue cultures or climate rooms are not regulated as strictly as in field production of GM food plants. However, there are opinion differences between EU and USA regarding the plant-made PMPs. In USA, regulation is at product level. Field use of GM plants and plants used in vaccine development are under secondary regulation of United States Department of Agriculture (USDA). In May, 2020 USDA Animal and Plant Health Inspection Service (APHIS) released revision on 7 Code of Federal Regulation (CFR) Part 340 regulations (85 Fed. Reg. 29790) which regulates the importation, interstate movement, and environmental release of genetically engineered organisms that are or may be plant pests [107]. Therefore, scrutiny on biotechnological production is further reduced in favor of researchers and companies. PMPs are currently under regulation of Title 21 (Food and Drugs) US CFR along with other traditionally produced counterparts. In EU regulation is on both production and final product level. All GM plant derived pharmaceuticals including plant based vaccines are under the same regulation of other biotechnology derived drugs which is indicated in European Commission Directive 2001/83/EC (on the Community code relating to medicinal products for human use) [108] and Regulation No 726/2004 (for the authorization and supervision of medicinal products for human and veterinary use) [109]. Also, if the host plant is a human food or animal feed source, cultivation and release of GM plant is under regulation of EC Directive 2001/18/EC (on the deliberate release into the environment of genetically modified organisms) [110] and 1829/2003/EC (on genetically modified food and feed).

Regulatory approval of a PMPs and other biotechnologically derived products may take up to a year depending on the legal response limits of the regulation agencies. However, Covid-19 pandemic triggered a realization on EU and UK for national and global needs. Therefore, product review, conditional approval and deployment timelines are significantly reduced for PMPs [111].

In conclusion, infectious diseases threatened humanity countless times throughout history. In particular, as pandemic and epidemic diseases killed millions of people, it increases in importance to develop safe and cost-effective vaccines and their storage and rapid distribution. Apart from many diseases such as diphtheria, cholera, typhoid, tuberculosis, which are controlled by vaccine campaigns in

developed countries, new vaccine production systems using recombinant DNA technologies are needed for emerging diseases such as COVID-19, MERS-CoV, avian influenza, Ebola, Zika and possible future infections. Plant-based vaccine production for humans and animals stands out as an important alternative that can be used to overcome the disadvantages of existing conventional vaccines. Within the scope of plant biotechnology, it became possible to produce cost-effective, immunogenic and safe vaccines thanks to the development of gene transfer strategies to plants and improvements in amount, isolation and purification and addition of adjuvant for production of recombinant vaccine antigens in plants. It is an undeniable fact that the possibilities that recombinant vaccines can offer us will increase with new standards and legal regulations to be introduced for the development, approval, authorization, licensing, distribution and marketing of such vaccines. In scope of future preventive healthcare, it is hard to assume monopoly of one particular vaccine technology. There will always be some ups and downs in any vaccine production methods. However, plant based vaccines represent considerable strong suits and offer swift and viable solutions over traditional and other recombinant vaccines.

Author details

Sinan Meriç*, Tamer Gümüş and Alp Ayan
Department of Molecular Biology and Genetic, Faculty of Science and Letters, Istanbul Kultur University, Ataköy, Istanbul, Turkey

*Address all correspondence to: s.meric@iku.edu.tr

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