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Review

Hepatitis B core-based virus-like particles: A platform for vaccine development in plants

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ABSTRACT

Virus-like particles (VLPs) are a class of structures formed by the self-assembly of viral capsid protein subunits and contain no infective viral genetic material. The Hepatitis B core (HBc) antigen is capable of assembling into VLPs that can elicit strong immune responses and has been licensed as a commercial vaccine against Hepatitis B. The HBc VLPs have also been employed as a platform for the presentation of foreign epitopes to the immune system and have been used to develop vaccines against, for example, influenza A and Foot-and-mouth disease. Plant expression systems are rapid, scalable and safe, and are capable of providing correct post-translational modifications and reducing upstream production costs. The production of HBc-based virus-like particles in plants would thus greatly increase the efficiency of vaccine production. This review investigates the application of plant-based HBc VLP as a platform for vaccine production.

1. Introduction

Due to the establishment of health organizations around the world, the level of health and well-being of people has increased in recent years. The mortality rate of infectious diseases has fallen and average life expectancy has increased. However, infectious diseases are still a major health concern of the World Health Organization (WHO) and other related organizations. In the 21 st century, various epidemics have been responsible for many deaths globally [1]. In recent years, the WHO has faced emerging dangerous viral diseases including SARS in 2002–2003, Influenza in 2009, MERS in 2012, Chikungunya in 2013, Ebola in 2014–2016, Zika in 2015-present, and SARS-CoV-2 (Covid-19) that has been recently (December 2019) identified in China has been declared a pandemic [2,3]. Therefore, in the case of epidemics, it is essential that a vaccine against the target pathogen be rapidly developed in large quantities. Traditionally vaccines have been composed of either live-attenuated or inactivated pathogens. These vaccines are efficacious but carry the risk of reversion to virulence. As an alternative, recombinant subunit vaccines, including virus-like particles (VLPs), are a new generation of vaccines that are not only safe but can also be rapidly produced in heterologous expression systems.

VLPs are essentially composed of structural proteins without incorporation of a genomic component and are thus non-infectious. A number of VLP-based vaccines against, for example, Hepatitis B virus and human papillomavirus are commercially available. The selection of an appropriate expression host is of crucial importance for such VLP-based vaccines [4,5]. Recombinant proteins used as biopharmaceuticals are complex molecules and thus require an appropriate host to attain the desired biological function. Factors that should be considered for the selection of an expression system include the correct folding and appropriate post-translational modifications of the protein, protein expression level and safety, contamination with endogenous agents, scalability, and production and maintenance costs. Recent advances in the development of expression systems have made it easier to select an appropriate system. Bacterial, yeast, insect, mammalian and plant expression systems all have specific advantages, as well as disadvantages, that need to be taken into account when selecting an expression system for a particular type of protein. Although easy to manipulate and scale-up, bacterial cells lack the post-translational machinery required for eukaryotic protein

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modifications and there is also the possibility of endotoxin and acetate accumulation, which cause detrimental effects on cell culture [6,7]. In insect cells, protein expression levels are often low and sites with potential N-linked glycosylation are often either glycosylated or not glycosylated, differing from glycosylation patterns in mammalian cells [8,9]. Although mammalian cells offer correct post-translational modification and protein folding, are scalable and yield adequate amounts of protein, limitations of this expression system include high costs of production, purification, and maintenance as well as safety issues with the possible contamination with endogenous pathogens. Due to an increased demand for large quantities of high-quality pharmaceuticals and diagnostic proteins in a limited amount of time and at low cost, the plant expression system is a suitable alternative with high potential in the production of recombinant vaccines and antibodies [10,11].

2. VLP-based vaccines

There are many types of biological and chemical agents for the prevention or control of disease, including monoclonal and polyclonal antibodies, peptides, small-molecule drugs, oligonucleotide-based therapeutics, interferons, and vaccines [12–14]. From the 18th century to the present time, great changes have taken place in the process of making vaccines. Vaccination has undoubtedly been one of the most successful and cost-effective health interventions, preventing the deaths of millions of people throughout the world every year. Most commercial vaccines are killed or live attenuated disease agents that induce immunity. However, one of the most critical problems of these vaccines is the possibility of reverting to virulence. New biotechnology and genetic engineering techniques have recently provided a viable, efficacious and, cost-effective alternative to these traditional vaccines. VLPs have been demonstrated to be safe, highly immunogenic and represent a promising new approach to vaccine development [15–19]. VLP vaccines were first developed in the early 1980s with the assembly of HBV VLPs in the yeast expression system [20]. A number of commercially available VLP-based vaccines are listed in Table 1 and include influenza [21] and hepatitis A vaccines [22]. VLP-based vaccines have all the characteristics of traditional vaccines capable of eliciting powerful and rapid cellular and humoral immune response [4] without the ability to replicate and cause disease. This is likely due to the preservation of the symmetrical size, shape, and structures of the infectious virus in the VLPs [23,24].

VLPs are regular nanometer-scale protein units with spontaneous assembly capability [25,26]. They are observed in a variety of viruses due to their size (22–150 nm). The structural proteins of VLPs spontaneously assemble after expression in recombinant systems [27,28]. These particles are often observed in the form of icosahedron or rod-like structures [29] and due to the absence of nucleic acid, they are non-replicating and not infectious [4]. There is thus no possibility of any genetic events occurring such as insertion, recombination, reversion, or re-assortment and therefore VLPs are generally considered safe. VLP vaccines, although viral in origin, have thus far not caused any serious side effects in inoculated individuals bar some classic mild adverse effects, such as local pain at the site of injection. These vaccines also have fewer side effects when compared to some other vaccines on the market. [18,30,31]. Another advantage of VLPs is that they are stable nanoparticles that can be utilized for the delivery of antigens or drugs [4,32,33]. There is also some evidence that the VLPs are less reliant on cold chains for storage and transportation [34,35]. Table 2 details VLP vaccine candidates undergoing preclinical or clinical trials (Table 3).

3. The immune response to VLPs

VLPs can induce both cellular and humoral immunity without the need for adjuvants [34]. They are able to trigger a potent T cell response [36–43].VLPs are absorbed by a variety of cells, including dendritic cells (DCs), due to their very small size, which is between 20−300 nm. It has been shown that adding sequences such as CpG can promote DC stimulation [44]. The internalization of VLPs by antigen-presenting cells (APCs) and subsequent presentation to CD8 + T cells on the MHC class I prompts cell activation, resulting in the induction of a potent immune response [42,45–47]. In addition, VLPs often display different pathogen-associated molecular patterns (PAMPs) that are recognized by pattern recognition receptors (PRRs) [23,48–51]. PAMPs induce immune responses through interaction with PRRs like Toll-like receptors (TLRs) on sentinel cells [23]. Furthermore, it is possible to insert several types of epitopes into the VLP at the same time using genetic engineering [44,52–54], which causes crosslinking between B cell receptors (BCR) leading to B cell activation [34,55]. Epitopes are also able to trigger a B-cell response [56]. Due to their repetitive and small structures, VLPs can prime the B cell for activation, i.e., multiplication and antibody production [57]. In this case, the antigens bound to MHC class II on the surface of APC interact with T helper cells eliciting IgG production and provide signals required

Table 1
Commercial VLP-based vaccines and their characterizations.

Vaccine Name	Pathogen	Antigen	Expression System	Company	Reference
Gardasil	HPV[1]	L1 protein 6,111,618	Yeast (*S. cerevisiae*)	Merck	[164]
Cervarix	HPV	L1 protein 1618	Insect/baculovirus	GSK	[165,166]
GenHevac B	HBV	PreS1 + 2 and HBsAg	Mammalian (CHO cells)	Pasteur-Merieux Aventis	[167]
Bio-Hep-B	HBV	HBsAg	Mammalian (CHO cells)	BTG (SciGen, FDS Pharma)	[168]
DTP-Hep B	HBV	HBsAg	Yeast (*P. pastoris*)	P.T. Bio Farma	[169]
Engerix-B	HBV	HBsAg	Yeast (*S. cerevisiae*)	GSK	[71,108]
Euvax B	HBV	HBsAg	Yeast (*S. cerevisiae*)	LG Life ScienceS	[170]
Gene Vac-B	HBV	HBsAg	Yeast (*H. polymorpha*)	Serum Inst. of India	[171]
Heberbiovac HB	HBV	HBsAg	Yeast (*P. pastoris*)	CIGB-Heber Biotec	[172]
Hepavax-Gene	HBV	HBsAg	Yeast (*H. polymorpha*)	Crucell	[173]
Recombivax HB	HBV	HBsAg	Yeast (*S. cerevisiae*)	MercK	[174,175]
Revac-B	HBV	HBsAg	Yeast (*P. pastoris*)	Bharat Biotech	[176]
Shanvac-B		HBsAg	Yeast (*P. pastoris*)	Shantha	[177]
Epaxal	HAV	Inactivated HAV RG-SB	Cell-free	Crucell	[22]
Inflexal V	Influenza	A (H1N1), A (H3N2), B, HA, NA	Cell-free	Crucell	[21]
Hecolin	HEV	capsid protein	*Escherichia coli* (Chinese market)	Xiamen Innovax Biotech Co	[178]

[1] Pathogen abbreviations.

Table 2
VLP-based vaccines that were expressed in different expression systems and their research phases.

Vaccine Name	Antigen	Stage of development	VLP type	Expression System	Sponsor	Reference
Chikungunya Virus	Glycoprotein	Phase I	Chikungunya Virus -VLP	Baculovirus	Jenner Institute, University of Oxford (UK)	[25,179]
Ebola virus	VP40, glycoprotein		Ebola virus-VLP	Insect cells	Animal Cell Technology Unit, IBET (Portugal)	[179,180]
Influenza A virus	haemagglutinin and matrix protein	Preclinical trials	Influenza A virus-VLP	Baculovirus	Instituto de TecnologiaQuímica e Biológica/ Universidade Nova de Lisboa, (Portugal)	[4,181]
Norovirus	NV capsid	Clinical trials	Norovirus-VLP	Escherichia coli	Jenner Institute, University of Oxford (UK)	[25,182]
Norwalk virus	Capsid	Phase I	Norwalk virus-VLP	Baculovirus	Animal Cell Technology Unit, IBET (Portugal)	[180,183]
Respiratory syncytiavirus (RSV)	G protein	Trials in non-human primates	Alfalfa mosaic virus-VLP	*Nicotiana tabacum*	Cell Biology Department, The Scripps Research Institute (USA)	[16,184]
Rotavirus	SA11 gene	Pre-clinical trials (Animal trials)	Rotavirus-VLP	Baculovirus	Cell Biology Department, The Scripps Research Institute (USA)	[16,185]
SARS-CoV-2	Spike glycoproteins	Phase I	CoVLP	*Nicotiana benthamiana*	Medicago, Quebec, QC, Canada	[186]
Influenza	H1N1	phase 3 trial	QVLP	*Nicotiana benthamiana*	Medicago, Quebec, QC, Canada	[187]

Table 3
Use of hepatitis B core as a vaccine platform to display epitopes in several expression systems.

Pathogen	Epitope(s)	Expression system	Site of epitope insertion	Ref.
Dengue virus	cEDIII	*N. benthamiana*	MIR	[68]
H1N1 Influenza A virus	matrix protein 2	*E. coli*	MIR	[188]
H7N9 Influenza	long alpha-helix (LAH)	*E. coli*	MIR	[189]
Mycobacterium Tuberculosis (Tuberculosis)	Culture filtrate protein 10 (CFP 10)	*E. coli*	MIR	[190]
–	Hepatitis B Core Antigen	*E. coli*	MIR	[86]
	GGS sequence	*Nicotiana benthamiana*		
Influenza virus	M2e	*E. coli*	MIR	[191]
Influenza virus	M2e	*N. benthamiana*	N-terminal	[192]
Dengue virus	EDIII-2	*E. coli*	MIR	[193]
Dengue virus	EDIII	*E. coli*	MIR	[193]
Hepatitis C virus	HCc N-terminus	*E. coli*	C-terminal	[194]
Influenza A	M2e	*E. coli*	N-terminal	[195]
Hepatocellular carcinoma	HBV X protein	*E. coli*	C-terminal, MIR	[196]
Hepatocellular carcinoma	AFP1, AFP2	*E. coli*	C-terminal	[197]
Hepatitis C virus	HCc T-cell epitope	*E. coli*	MIR	[198]
Foot-and-mouth-disease virus	VP1, VP4	*N. tabacum, E. coli*	MIR	[199,200]
Hepatitis B virus	Pre-S1	*E. coli*	MIR	[201]
Hantavirus	Nucleocapsid protein	*E. coli*	MIR	[202]
Hepatitis B virus	Pre-S1	*E. coli*	C-terminal	[203]
Human Papillomavirus	E7	*E. coli*	C-terminal	[204]
Theileria annulata	SPAG-1	*E. coli*	MIR	[205]
Human Immunodeficiency Virus	Gag	*E. coli*	N-terminal	[206]
Human Immunodeficiency Virus	Env	*E. coli*	C-terminal	[90]
Foot-and-mouth-disease virus	VP1	*E. coli*	N-terminal	[67]

for differentiation of B cells toward memory B cells. VLPs are often delivered without an adjuvant [57], leading to the production of high antibody titers [55,58].

4. Engineering VLPs as a vaccine platform

The regular structured VLPs have made it possible to insert and present heterologous epitopes on the surface of these particulate structures [16]. HPV16 L1 protein, which self-assembled into VLPs in plants, has proven highly immunogenic and efficacious for vaccine production [59]. Also very recently, the use of grapevine fanleaf virus (GFLV) VLPs has been studied as a new carrier for the presentation of the HPV L2 epitope [60]. More than 40 years have passed since VLPs were first obtained from the HBV surface antigen of HBsAg [40,61]. The HBcAg polypeptide is about 21 kDa in size and consists of 183–185 amino acid residues that can self-assemble into 27-nm particles [62–64]. HBc VLPs have been widely studied in the last two decades. They were first reported in 1987 as carriers displaying heterologous epitopes of foot and mouth disease virus. After the primary study by Clarke et al. in 1987 [65], different epitopes and antigens have been introduced into HBc protein for vaccine development (Table 6). The immunogenic epitope of the virus was fused to the N-terminus end of the HBc sequence [66–69]. This VLP has been used to produce HBV vaccine in yeast [70,71] and mammalian cells (CHO) [20]. HBc has been expressed in various prokaryotic and eukaryotic expression systems [20,36,42,43,70,72–85]. X-ray crystallography and cryo-electron microscopy studies on HBc components revealed that these are icosahedral particles [86]. HBc monomers are observed in two different sizes when packaged as a VLP, consisting of 180 or 240 subunits, and their symmetry is obtained in two forms, T = 3 or T = 4 [87]. The linear structure of the protein consists of two parts. Residual amino acids 1–140 comprise the N-terminus region, which consists of a SA domain required for self-assembly. The C-

terminus (CTD), is a region (150–183 amino acids) rich in arginine called protamine [88]. Gallina et al. in 1989 showed that the protamine domain is not related to the particle assembly, but it is important for stabilizing the particles with neighboring disulfide bonds [89]. SA domain has a variable area associated with B-cell epitopes, while the second CTD and hinge peptide are highly conserved [78]. The N-terminus in HBcAg is used as an insertion site for external epitopes, which enable the insertion of up to 50 amino acids and induce specific antibody responses. Another place of insertion is the end of the C terminus and in the position of amino acids 144−127. In the case of the C-terminus, it is possible to insert the epitopes at amino acids 144, 149, 153, 163, and 169 [66]. In 1989, Stahl and Murray successfully fused the immunogenic part of HBsAg of HBV and the envelope of Human Immunodeficiency Virus (HIV) to the C-terminus of HBc sequence [90].

In addition to the N and C termini of the HBc protein, epitopes may also be inserted into the Major immunodominant region (MIR) in the e1 loop of the HBcAg protein [13]. Studies have indicated that such insertions result in a 10-fold stronger immune response and may be the most efficient site for epitope presentation [91,92]. The position of the MIR region is located between the amino acids 82–88 and at the tip of the α-coil [77]. Four cysteines exist in the HBcAg sequence located at 48, 61, 107, and 183 positions. Disulfide bonding between two monomers to create a dimer usually takes place between cys 61 and sometimes cys 48 of adjacent monomers [86]. The icosahedral VLPs are formed by the association of 180 (90 dimers; T = 3) or 240 (120 dimers; T = 4) copies of HBcAg proteins. VLPs with 180 HBcAg copies are about 30 nm, while those with 240 copies are approximately 34 nm in diameter [93]. The dimer consists of four-helix bundles connected by loops that form spikes on the outer surface of VLPs. The amino acid residues 76–82 of each HBcAg monomer known as MIR or c/e1 loop, are placed at the tip of the spike after VLP assembly. Therefore, after inserting an epitope into the MIR or C- or N-termini regions of an HBcAg monomer, HBc VLPs can self-assemble following the expression of the monomeric form [68,69,92]. HBc VLPs was one of the first VLPs produced in the plant system through transient expression technology [76] Recently, Peyret et al. 2015, have introduced a system called the 'Tandem Core', which is shown in Fig. 1, and which has advantages over the previous system including the reduction of steric clashes due to the non-random association of subunits, flexibility in inserting a wide range of epitopes, and flexibility to insert larger epitopes. It has been shown that inserting large or hydrophobic sequences in the MIR region can reduce antigenicity and immunogenicity [77]. One way to overcome this problem is to create a mosaic VLP so that HBcAg proteins with large insertions in their MIR area are co-expressed with wild-type HBcAg [94]. Another solution is to use a 'Split Core' and insert an antigen into the N- and C-termini regions [86] thus creating a dimer protein. Particles have high inherent antigenicity due to their spiky and repetitive structures that allow the presentation of antigens on their surface [78,95]. Because of its polymeric nature and the presence of a large number of T-cell epitopes, HBc protein has high immunogenicity [95]. HBc protein exists in two forms, T-cell-dependent and T-cell-independent. They can activate macrophages, and high antibody production [96–99]. HBc, as a potent T cell epitope, can stimulate Toll-like receptors [100,101]. These antigens can act as T-cell and B-cell epitopes and induce immunogenicity [102–104]. HBc VLPs are very immunogenic in laboratory models such as mice and no cytotoxic effects have been reported in humans. [69]. Recently HBc was suggested as a new platform against SARS-CoV-2 by exposing immunogenic epitopes [105].

5. Expression systems used for VLP-based vaccine production

Despite the commercialization of several VLP-based vaccines, the current bacterial, yeast, insect and mammalian cell expression systems suffer from various limitations [106–108]. As the selection of a suitable expression system results in increased vaccine efficacy, scalability, and performance, and can affect the production costs, it is important that the advantages and disadvantages of each system be weighed for each particular vaccine candidate. A comparison of the differences between the available expression systems is given in Table 4. Bacteria are the most commonly used expression system for the production of recombinant proteins, and 30 % of the VLPs described are produced in the bacteria [109]. Interestingly, despite the high number of VLPs produced in *E. coli*, no VLP derived from this bacterium has yet been commercialized. The main concerns are the inability of prokaryotes to perform post-translational modifications and the complexity of protein

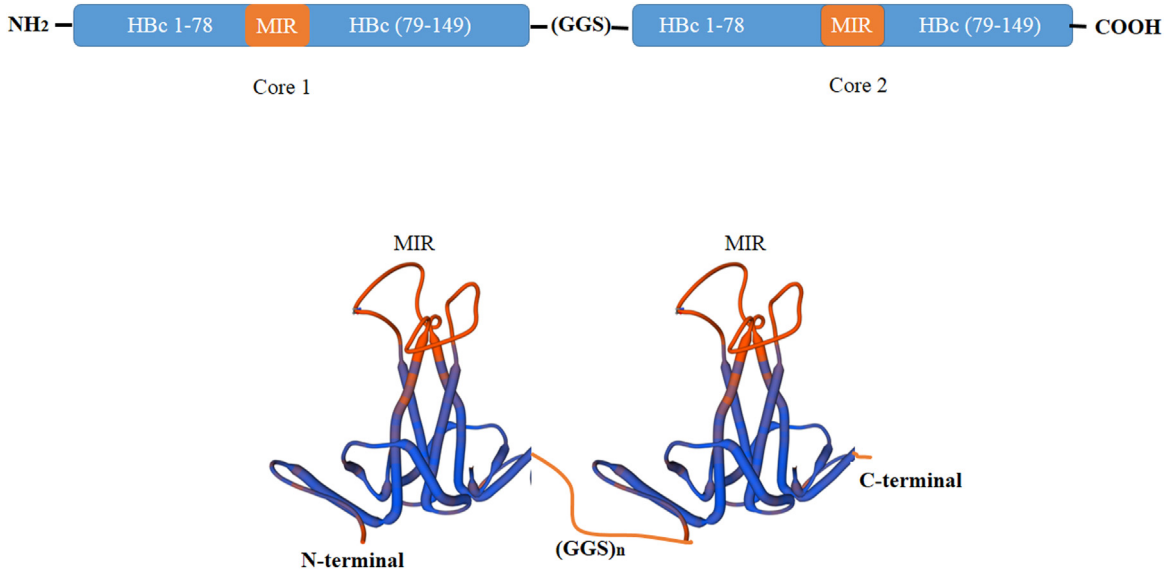

Fig. 1. Illustration of the Tandem Core technology concept. The image shows two HBcAg genetically fused to one another via a flexible linker. MIR: Major immunodominant region.

Table 4
Comparison of different expression systems for the generation of pharmaceutical proteins.

Disadvantages	Advantages	Production method
✓ Lack glycosylation. ✓ Endotoxins ✓ Low Protein folding accuracy and export ✓ Product size limitations ✓ May require protein-specific optimization	✓ Ease of expression ✓ Ability to scale-up ✓ Low production cost ✓ Low maintenance costs ✓ Simplicity of genetic manipulation	***E. coli***
✓ Non-appropriate protein glycosylation. ✓ Risk of incorrect folding & assembly ✓ Medium protein folding accuracy ✓ Fermentation require for very high yield	✓ Ease of expression ✓ Ability to scale-up ✓ Low production cost ✓ Low maintenance costs ✓ Eukaryotic protein processing	**Yeast**
✓ High mannose glycoprotein modification ✓ Difficulty eliminating pollution caused by baculovirus ✓ Unsought posttranslational modifications ✓ Low yields compared to the bacterial and yeast systems	✓ Produce large amounts of VLP in high density cell culture conditions. ✓ Minimal risk of opportunistic pathogens ✓ Establish a stronger immune response through the cellular components of baculoviruses ✓ Average maintenance cost ✓ High protein folding accuracy ✓ Similar mammalian protein processing	**Insect cells**
✓ Higher production cost ✓ Lower productivities ✓ Heterologous output ✓ High risk of human/ /animal pathogen ✓ Prolonged production efficiency	✓ Producer cells more closely related to the natural host ✓ Appropriate PTMs and authentic assembly of VLP ✓ High maintenance costs ✓ High protein folding accuracy ✓ High yields	**Mammalian cells**
✓ Low expression levels (transient expression systems showed high expression levels)	✓ Ease of expression ✓ Ability to scale-up ✓ Low production cost ✓ Low maintenance costs ✓ High protein folding accuracy ✓ Optimal growth stages ✓ Lack of pathogenic risk in humans ✓ Good secretion ✓ stability of product	**Plants**

purification owing to the significant differences between the eukaryotes and the prokaryotes [110–112]. Yeast, insect and mammalian cells have also been extensively utilized in the production of VLPs [113,114]. Yeast expression systems are the most utilized in recombinant vaccine production due to the relative ease of genetic manipulation and their rapid growth. Yeast cells are most effective in producing non-enveloped VLPs and also make the study of complex VLPs possible [106,115,116]. However, the glycosylation capacity of yeast cells is limited [117]. Other disadvantages of the yeast expression system include low yield, low plasmid stability, and low secretion capacity [118,119]. In the case of insect cells, post-translational changes can be accompanied by high levels of sugars resulting in hyper glycosylation, which affects the effectiveness of the vaccine and increases the cost [120–123]. Furthermore, protein expression in both bacterial and yeast systems is lower than in plants [124]. There are some disadvantages in using of insect expression system including the possibility of contamination of insect cell cultures, different post-translational modifications and proteolysis cleavage in areas rich in Lysine and Arginine. Mammalian expression systems have advantages such as correct post-translational modifications, correct protein folding and are free of bacterial endotoxins. However, the need for special fermentation devices, time-consuming cell preparation, slow growth, the possibility of contamination with endogenous pathogens, and high production and maintenance costs limit its use in many countries, especially developing countries [125]. Plants are considered a cost-effective, scalable, efficient, and safe alternative to the current mainstream expression systems for the production of VLP-based vaccines.

6. Plants as expression systems for VLP-based vaccine production

Over the last two decades, plants have been increasingly used as a production host for recombinant proteins. Plant expression systems are characterized by a high yield of proteins, ease of protein purification and faster recombinant protein production [126–129]. A major advantage of the plant expression systems is their ability to produce large amounts of recombinant protein in a fast and cost-effective manner [130–132]. Whole plants can be grown in large quantities in greenhouses without requiring bioreactor-based fermentation methods [133]. In plants, it is only necessary to increase the area under plant cultivation to increase antigen production [124]. Plants have a short growth cycle and mature quickly, thus reducing the production costs compared to other systems. Hiatt et al., in 1989 produced the first monoclonal antibody (mAb) in the transgenic tobacco plant [134]. Examples of VLP-based vaccines produced in plant expression systems include those against Influenza and foot-and-mouth diseases [135–138]. Plant virus nanoparticles have also been used as carrier particles for drug delivery and imaging [139]. Although the glycosylation patterns in plants are slightly different from those of mammalian cells [65,140], plant-expressed H5N1 influenza virus-like particles have demonstrated safety in Phase II clinical trials [141,142] and plant-made pharmaceuticals are aggressively being investigated, most recently against the Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). A phase 1 trial of a VLP-based vaccine against SARS-CoV-2, transiently expressed in plants, was recently conducted by Medicago [143]. In plants, there is also no need to

Table 5
Plant-derived vaccines and their clinical trial phase.

Target	Interventions?	Plant Host	Study Type	Study Completion Date	Sponsor
Malaria	Pfs25 VLP- FhCMB	*N. benthamiana*	Interventional (Clinical Trial: Phase 1) (NCT02013687)	January 2015	Fraunhofer, Center for Molecular Biotechnology
Anthrax	PA83-FhCMB	*N. benthamiana*	Interventional (Clinical Trial: Phase 1) (NCT02239172)	May 2015	Fraunhofer, Center for Molecular Biotechnology
Influenza A Subtype H5N1 Infection	H5-VLP + GLA-AF; Licensed H5N1 vaccine	*N. benthamiana*	Interventional (Clinical Trial: Phase 1) (NCT01657929)	January 2014	IDRI
H5N1 Flu	HAI-05 Influenza Vaccine; Saline	*N. benthamiana*	Interventional (Clinical Trial: Phase 1) (NCT01250795)	July 2011	Fraunhofer, Center for Molecular Biotechnology
2018–2019 influenza season	Quadrivalent VLP Vaccine	*N. benthamiana*	Interventional (Clinical Trial: Phase 3) (NCT03739112)	June 14, 2019	Medicago
H1N1 Flu	HAC1 Vaccine	*N. benthamiana*	Interventional (Clinical Trial: Phase 1) (NCT01177202)	October 2012	Fraunhofer, Center for Molecular Biotechnology
H1N1 Flu	Quadrivalent VLP Vaccine; Placebo	*N. benthamiana*	Interventional (Clinical Trial: Phase 3) (NCT03301051)	June 2018	Medicago
H1N1 Flu	H1N1 VLP vaccine	*N. benthamiana*	Interventional (Clinical Trial; phas 1) (NCT01302990)	July 2011	Medicago
H5N1 Flu	H5N1 VLP vaccine	*N. benthamiana*	Interventional (Clinical Trial; Phase 2) (NCT01991561)	July 2014	Medicago
H7N9 Flu	H7N9 VLP vaccine	*N. benthamiana*	Interventional (Clinical Trial; Phase 1) (NCT02022163)	September 2014	Medicago
Lymphoma, Follicular	Autologous FL vaccine	*N. benthamiana*	Interventional (Clinical Trial; Phase 1) (NCT01022255)	October 2013	Icon Genetics GmbH
HIV Infection	P2G12	*N. tabacum*	Interventional (Clinical Trial; Phase 1) (NCT02923999)	August 2020	St George's, University of London

Table 6
VLP-based vaccines produced in plants.

Application	Antigen	Production Plant	Study Phase	Sponsor	Reference
Bluetongue	VP3,VP7, VP5 and VP2 protein	*N. benthamiana*	Pre-clinical	Department of Biological Chemistry, John Innes Centre, Norwich, UK	[207]
Foot-and-mouth disease	Structural proteins (VP0, VP1 and VP3)	*N. benthamiana*	Research	Institute of Infectious Disease and Molecular Medicine (South Africa)	[208]
Hepatitis B	HBsAg	Tobacco	Phase I	Biodesign Institute at Arizona State University (USA)	[209]
Hepatitis B	HBsAg	Lettuce	Phase I	Institute of Biotechnology and Antibiotics (Poland)	[210,211]
Hepatitis B	Glycol protein	Spinach	Phase I	Institute of Biotechnology and Antibiotics (Poland)	[212]
HIV	Pr55 gag protein	Tobacco	Research	Institute of Plant Genetics (Italy)	[213]
HBV-HIV	env and gag proteins	Tomato	Research	State Research Center of Virology and Biotechnology Vector (Russia)	[214]
HPV	16 L1	*Nicotiana tabacum*	Research	Department of Molecular and Cell Biology (South Africa)	[215]
Influenza	HA(H5N1)	*N. benthamiana*	Phase I/II	Medicago (Canada)	[216,217]
Influenza	HA(H1N1)	*N. benthamiana*	Phase I	Medicago (Canada)	[218]
Influenza	HA(H5N1)	*N. tabacum*	Phase II	Infectious Disease Research Institute, Seattle, WA (USA)	[142]
Influenza	H5N1	*N. benthamiana*	Research	Department of Research & Development, Haukeland University Hospital, Bergen, (Norway)	[219]
Malaria	Pfs25-CP	*N.Tobacco*	Phase I	Center for Molecular Biotechnology, Plymouth, MI(USA)	[220]
Noroviruses	NaVCP	*N. benthamiana*	Research	Center for Infectious Diseases and Vaccinology (USA)	[221]
Norwalk	Capsid protein	Potato and tobacco	Phase I	University of Maryland (USA)	[222]
Norwalk	Capsid proteins	Tomato	Pre-clinical	Biodesign Institute and School of Life Sciences (USA)	[150]

worry about the contamination of toxins and endogenous pathogens, which usually occur in bacterial and mammalian cell-based expression systems [144–146]. Unlike prokaryotic systems, plants have the same secretory pathway as human cells [147]. Another feature of this expression system is the simplicity of storing the recombinant proteins [148,149]. Table 5 shows the list of vaccines produced in plants.

Although plants such as potatoes, tomatoes, corn, soybeans, rice, and carrots have been used to study immunogenicity, particularly that of oral vaccines that trigger the mucosal

immune response [150–152], *Nicotiana benthamiana* plants are the stalwart of plant expression system and offer a number of advantages, including non-food crop status, high growth rate, growth in greenhouses that reduces the risk of spread of contamination, and accessibility of suitable and efficient vectors for enhanced gene expression [153–156]. As indicated in Table 6, diverse types of VLPs have been expressed in plants. Expression in these plants can be either transient, which enables rapid, high-volume and low-cost production of vaccines [145], or transgenic, which, although stable, typically yields low levels of expression compared to the transient expression [157]. The transient expression makes screening and production of the desired protein possible in a few days which is particularly important for the development of vaccines during epidemics. Protein expression is also limited to the infiltrated tissue preventing the risk of transfer of foreign genes to the gametes of the plant, thus inhibiting cross-pollination and spread within an unintended plant population. Protein production on a large scale can be accomplished by means of vacuum infiltration of all plant leaves [158]. Plant cell culture has emerged as an alternative bio-production system for the production of recombinant pharmaceuticals. The recombinant glucocerebrosidase enzyme (Taliglucerase Alfa) that is produced within carrot cells, has been developed as a plant cell-made pharmaceutical for the treatment of Gaucher's disease [159].

In transgenic products, the production of vaccines can be directed into the cytoplasm, as it has been done in tobacco and lettuce [160,161]. In a study, the insertion of some epitopes of *T. gondii* into a truncated HBcΔ particle triggered strong humoral and cellular immune responses [162]. A more immunogenic VLP vaccine containing, in addition to HBcAg, the proteins PreS1 and PreS2, was found to elicit a strong antibody response [163]. Like VLPs produced in other systems, some plant-derived VLPs are able to induce protective humoral and cellular immune responses. Plant-expression systems can produce large quantities of immunogenic HBcAg [24]. Several recombinant VLPs, including HBcAg VLPs displaying M2 epitope of influenza A, yeast transposon Ty VLPs displaying HIV p17/p24 antigens, and HBcAg VLPs displaying malaria epitopes have been evaluated. Also, expression and assembly of HBcAg-HPV16 L2 epitope VLPs in tobacco-induced antigen-specific antibody response in mice [24].

7. Conclusion

Many virus-like particles have been used in the last three decades experimentally or commercially for various purposes such as drug loading and delivery, medical imaging and vaccine production. Hepatitis B virus (HBV) core particles (HBc) have been the VLP of choice for commercial vaccine production, not only against HBV but also against many other viral and non-viral agents. The HBsAg particle was the first VLP-based vaccine and today, due to its compatibility with a diverse array of expression systems, many of these particles are manufactured for commercial purposes. HBc is also one of the most promising VLP presentation platforms due to its high immunogenicity, its enhanced presentation to the immune system, and its flexibility to allow a wide variety of foreign insertions without affecting the protein self-assembly and VLP function. These features will increase the versatility and efficiency of this type of vaccine in the future. The production of HBc VLP-based vaccines in plants is an attractive option in developing countries where the implementation of more sophisticated technologies is often met with difficulties. The use of HBc and other VLP platforms, including those of plant viruses, is a developing field in biopharmaceutical and medical sciences.

Declaration of Competing Interest

The authors declare that they have no conflict of interest. The research reported here did not involve experimentation with human participants or animals.

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