



Recombinant helical plant virus-based nanoparticles for vaccination and immunotherapy

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Abstract

Plant virus-based nanoparticles (PVNs) are self-assembled capsid proteins of plant viruses, and can be virus-like nanoparticles (VLPs) or virus nanoparticles (VNPs). Plant viruses showing helical capsid symmetry are used as a versatile platform for the presentation of multiple copies of well-arrayed immunogenic antigens of various disease pathogens. Helical PVNs are non-infectious, biocompatible, and naturally immunogenic, and thus, they are suitable antigen carriers for vaccine production and can trigger humoral and/or cellular immune responses. Furthermore, recombinant PVNs as vaccines and adjuvants can be expressed in prokaryotic and eukaryotic systems, and plant expression systems can be used to produce cost-effective antigenic peptides on the surfaces of recombinant helical PVNs. This review discusses various recombinant helical PVNs based on different plant viral capsid shells that have been developed as prophylactic and/or therapeutic vaccines against bacterial, viral, and protozoal diseases, and cancer.

Keywords Helical plant virus · Coat protein · Nanoparticle · Antigens · Vaccine · Immunotherapy

Introduction

Edward Jenner was the first to develop a vaccine against smallpox in 1796, and subsequently, inactivated and attenuated pathogens were used as vaccines. However, due to the difficulties associated with the propagation of pathogens in vitro and the reversion to virulence, subunit vaccines and conjugate vaccines were developed to provide alternative, safer approaches to inactivated and attenuated vaccination [1]. Recombinant DNA technology has been harnessed to develop subunit vaccines against pathogens using various expression systems, such as, *Escherichia coli*, yeast, mammalian, and insect cell lines. However, the increased need for

cheaper vaccines remains a challenge in developing countries because of economic and logistical problems [2].

To overcome these constraints, plants can be used as low-cost biofactories to produce vaccines at commercial scales. Several plant species, such as, tobacco, spinach, lettuce, and tomato, have been used to produce vaccines, and the antigenic proteins and peptides derived from plant biomasses or purified fractions have been used as vaccines to elicit protective immunity against animal pathogens [3]. Most animal pathogens enter the body through mucosal tissues or infect directly at mucosal sites. The oral administrations of plant-based mucosal vaccines can induce humoral and cell-mediated immune responses, and this form of delivery to mucosal surfaces also makes immunization safe and eliminates needle-associated risks [4]. Since these recombinant subunit vaccines contain only immunogenic epitopes of pathogens, their immunogenic properties are substantially less than those of whole pathogens, and thus, adjuvant co-administration is needed to induce higher immune responses [5]. However, an alternative strategy to increase the immune response is to arrange multiple copies of immunogenic epitopes in a well-ordered and defined manner on a nanoscaffold.

Self-assembling coat proteins from viruses form the protein nanostructures of the parent virus, can be free of genetic

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material, and are referred to as virus-like particles (VLPs). These protein nanostructures or nanoscaffolds mimic viruses, but are non-infectious and lack replication potential [6]. Accordingly, they can be used as carriers of antigenic epitopes to elicit effective immune responses. VLPs with replication potential in plants are virus nanoparticles (VNPs). These VNPs and VLPs known as plant virus-based nanoparticles (PVNs) can be made from isometric or helical viruses, and can be genetically engineered with antigenic epitopes from pathogens to form recombinant isometric or helical PVNs (rPVNs) for the safe and effective elicitation of immune responses [5, 7–9]. In this review, we discuss reports on recombinant helical PVNs used as vaccines against various pathogens and in cancer immunotherapy.

Helical plant viruses

Viruses are submicroscopic, non-cellular, obligate intracellular parasites, which cannot grow or reproduce by themselves, and require host cell machinery to replicate. They consist of a protein coat (capsid), which encloses genetic material (DNA or RNA). Viruses are host-specific and are categorized based on the host organisms as bacteriophages, cyanophages, phytophages, or zoophages. Phytophages are plant viruses, which cause diseases in plants with a range of symptoms, particularly, leaf yellowing, and growth distortions [10]. They are classified by their nucleotide sequence, genome expression strategy, mode of transmission, and particle structure. Viruses are also classified by capsid design as icosahedral and helical. Icosahedral-shaped viruses are roughly spherical shaped, while helical viruses are filamentous. Moreover, depending on the presence or absence of a lipid bilayer around the capsid, viruses are classified as enveloped or non-enveloped. Non-enveloped helical plant viral capsids are more flexible and stable in terms of expressing foreign genes, which makes them an ideal platform for epitope presentation system [11] (Fig. 1).

Genetic engineering of helical plant virus-based nanoparticles (PVNs)

Helical PVNs are rod shaped and their N- or C-terminal coat proteins (CPs) are exposed on their surfaces. Furthermore, CPs have been fused with antigenic epitopes of disease pathogens by genetic engineering to elicit immunogenic activity. In addition, genetic modifications of surface amino acids of PVN CPs to lysine or cysteine allow bioconjugation with antigenic peptide or protein entities [12]. In particular, helical plant viruses, such as, bamboo mosaic virus (BaMV), cardamom mosaic virus (CdMV), johnsongrass mosaic virus (JGMV), papaya mosaic virus (PapMV), papaya ringspot virus (PRSV), plum pox potyvirus (PPV), potato virus X (PVX), potato virus Y (PVY), tobacco etch virus (TEV),

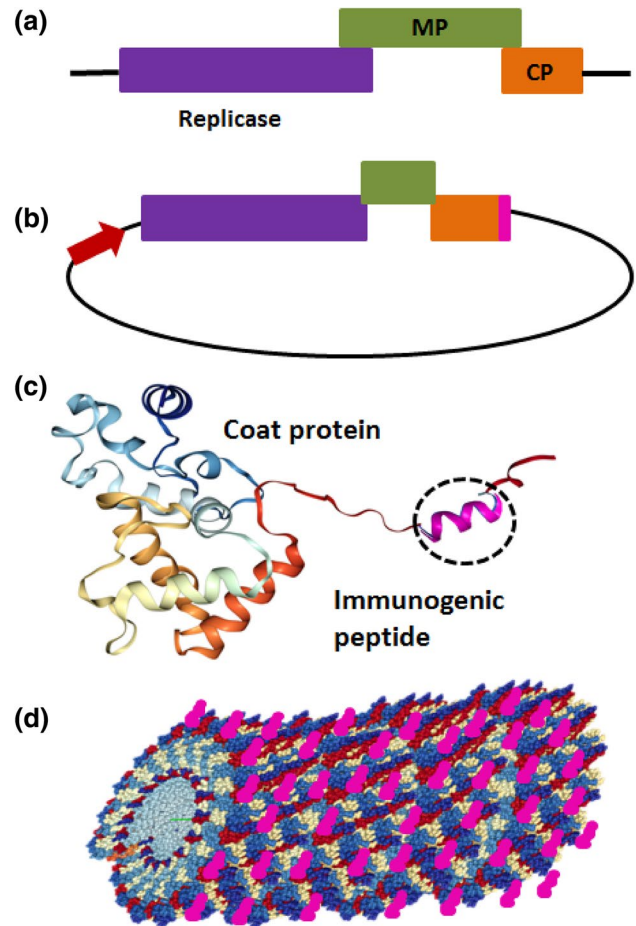


Fig. 1 Self-assembly of helical virus-based nanoparticles from recombinant coat proteins containing immunogenic epitopes: **a** schematic diagram of the helical plant viral genome, **b** plant virus-based expression vector containing an immunogenic epitope as a fusion protein, **c** recombinant coat protein containing an immunogenic epitope, and **d** recombinant helical plant virus-based nanoparticles displaying immunogenic epitopes

tobacco mosaic virus (TMV), and zucchini yellow mosaic virus (ZYMV) have been genetically engineered to display immunogenic epitopes on their surfaces to provide effective vaccination against several disease pathogens and for immunotherapy (Table 1).

Bamboo mosaic virus (BaMV)

Bamboo mosaic virus (BaMV) is a flexuous, rod-shaped, non-enveloped virus of width 15 nm and of clear modal length 490 nm, and contains a positive-sense single-stranded RNA (ssRNA). It is a member of the *Potexvirus* genus and infects both mono- and dicotyledonous plants [13, 14].

Many helical plant viruses, such as TMV and PVX, are used as versatile vectors for the production of vaccines by expressing antigenic and immunogenic epitopes in plants.

Table 1 List of recombinant helical plant virus-based nanoparticles (rPVNs) functionalized with immunogenic peptides display for vaccination

Recombinant helical PVNs	Vaccination	Immunogenic epitope display	References
1. Bamboo mosaic virus (BaMV)	Foot-and-mouth disease virus	VP1 capsid protein (T ¹²⁸ -N ¹⁶⁴)	[16]
2. Cardamom mosaic virus (CdMV)	Human immunodeficiency virus	Envelope glycoprotein gp41	[21]
	<i>Leptospira interrogans</i>	Membrane protein LipL32	[22]
3. Johnsongrass mosaic virus (JGMV)	<i>Plasmodium falciparum</i>	Merozoite surface antigen (MSA2)	[27]
	<i>Schistosoma japonicum</i>	Sj26-glutathione S-transferase	[28]
	Japanese encephalitis	Peptide A _{373–399} of Envelope (E) protein	[30]
	Contraception	YLP12 testes-specific peptide & ZP3 peptide	[33]
4. Papaya mosaic virus (PapMV)	Hepatitis C virus	Decapeptide of LHRH	[34, 35]
	Influenza virus	E2 glycoprotein	[38]
		Extracellular domain of M2e epitope	[39]
		M1 matrix protein	[40]
5. Papaya ringspot virus (PRSV)		Nucleocapsid (NP _{147–155})	[43]
	Canine parvovirus	VP2 capsid peptide	[50]
	Dengue virus	Domain III of DENV2E protein	[52]
6. Plum pox potyvirus (PPV)	Canine parvovirus	VP2 capsid protein	[55, 56]
7. Potato virus X (PVX)	Human immunodeficiency virus	Glycoprotein 41 (ELDKWA peptide)	[62]
8. Potato virus Y (PVY)	Hepatitis B virus	preS1 epitope	[67]
9. Tobacco etch virus (TEV)	Porcine reproductive and respiratory syndrome virus (PRRSV)	Minor and major envelope proteins	[71]
10. Tobacco mosaic virus (TMV)	Human immunodeficiency virus	HIV-1 Tat protein	[77]
		Envelope protein gp120	[78]
	Influenza virus	Hemagglutinin (HA) glycoprotein	[78]
	Canine oral papilloma virus	L2 protein (L2 _{61–71})	[79]
	Murine hepatitis virus	S2 glycoprotein	[89]
		5B19 epitope of spike (S) glycoprotein	[90]
	Rabies virus	G5-24 peptide of virus glycoprotein	[90]
		Peptide 31D of nucleocapsid protein	[90]
	Poliovirus	Peptide from VP1 capsid protein	[92]
	Foot-and-mouth disease virus	F11 & F14 peptides from VP1 capsid protein	[93, 94]
	<i>Francisella tularensis</i>	OmpA-like protein (OmpA)	[95]
		Chaperone protein DnaK	[95]
		Lipoprotein Tul4	[95]
	<i>Pseudomonas aeruginosa</i>	OM protein F (9–14 mer peptide)	[97]
	<i>Plasmodium falciparum</i>	B-cell epitope from CSP	[98]
	<i>Plasmodium yoelii</i>	Merozoite surface protein 4/5	[99]
	Cancer	8-aa mouse tumor T-cell epitope	[101]
		Tn (GalNAc- α -O-Ser/Thr) antigen	[102]
		14- or 36-aa HER2/neu epitope	[103]
	Contraception	ZP3 glycoprotein of zona pellucida	[104]

However, helical BaMV is a less-explored viral vaccine delivery system that can carry larger transgene loads and generate better immunity against various diseases in target animals with fewer adverse effects. Foot-and-mouth disease (FMD) is caused by foot-and-mouth disease virus (FMDV), which has seven major serotypes (O, A, C, SAT-1, SAT-2, SAT-3, and Asia-1), and infects cloven-hoofed animals, such as pigs, sheep and cattle, causing serious damage in the livestock industry [15]. Yang et al. [16] generated a recombinant

BaMV-based vector (pBVP1) for the production of chimeric BaMV virus in plants as a vaccine against FMDV. pBVP1 was constructed by inserting a DNA sequence encoding 37 amino acid residues (T¹²⁸-N¹⁶⁴) of the VP1 CP of FMDV (O/Taiwan/97) by replacing the DNA sequence encoding BaMV CP. When transfected into *Chenopodium quinoa*, pBVP1 generated chimeric BVP1 virions expressing antigenic epitope(s) of the capsid protein VP1 of FMDV in its coat protein. Immunization of swine with BVP1 virions as

VLPs resulted in the production of anti-FMDV neutralizing antibodies. Thus, chimeric BVP1 virions expressing a partial sequence of VP1 protected target animals by generating humoral and cell-mediated immune responses, and conferred full protection against FMDV.

Cardamom mosaic virus (CdMV)

The filamentous plant viruses of the family *Potyviridae*, which include genus *Potyvirus*, *Rymovirus*, *Tritimovirus*, *Bymovirus*, *Maclurovirus*, *Ipomovirus*, and *Brambyvirus*, constitute almost a quarter of the known plant viruses [17]. Among these, Potyviruses are flexuous, rod-shaped particles, 700–900 nm long and 11–15 nm wide with a single copy of a positive-sense polyadenylated ssRNA of about 10 kb. A mature potyvirus particle contains approximately 2000 copies of a single type of CP in the range of 28–40 kDa. Chimeric potyvirus-like particles (PVLPs) can be made by fusing foreign sequences to the N- and/or C-terminal regions of the CP, which are surface-exposed and immunodominant and are not required for the self-assembly of PVLPs [18].

Cardamom mosaic virus (CdMV) is a member of the genus *Macluravirus* of *Potyviridae* and the causative agent of cardamom mosaic disease infecting small cardamom (*Elettaria cardamomum* Maton) with mosaic, katte or marble disease [19, 20]. Virions are filamentous, usually flexuous of 700–720 nm, and transmitted by aphids in a non-persistent manner, and not transmitted by mechanical inoculation and seed. The CP of CdMV was used as a carrier molecule for presenting HIV-1 antigens. The envelope glycoprotein gp41 of HIV plays an important role in viral entry into the host cells. The Kennedy peptide, contains three epitopes (aa 735–752), is located in the cytoplasmic tail of gp41. The epitopes, 2F5, which is at the end of the heptad repeat 2 region of gp41, and 4E10 at the C-terminus of the 2F5 binding region, neutralize the virus by interfering with its fusion with the target cell membrane. The N- and C-termini of the CdMV-CP were engineered with the Kennedy peptide (E1) and the 2F5 (ELDKWA) and 4E10 epitopes of gp41 of HIV. These chimeric proteins had the ability to react with sera from HIV-infected persons and to induce cytokines in their PBMCs [21]. Similarly, molecular modeling and *in-silico* analyses showed that surface-associated epitopes, LipL32 of *Leptospira interrogans*, can be displayed on the N-terminus of CdMV-CP for the development of Leptospirosis vaccine. Antibody developed against these LIPL32 epitopic regions could also be used for the detection of Leptospirosis [22].

Johnsongrass mosaic virus (JGMV)

Johnsongrass mosaic virus (JGMV) is a plant pathogenic virus of the family potyviridae, which was first reported in *Sorghum halepense* (Johnsongrass) and *Zea mays* from

Australia [23]. It was transmitted by aphids, mechanical inoculation and not transmitted by contact between plants and seeds. JGMV-infected plants, mostly show systemic mosaics, mottles, ringspots, or necrosis. It is usually a flexuous filamentous virion with a clear modal length of 773–778 nm, with the CP gene coding for a protein of 303 amino acids with a molecular weight of 34 kDa [24].

Vaccines for human parasitic diseases

RTS,S/AS01 (Mosquirix) is the first VLP-based vaccine generated against malarial parasitic disease using the circumsporozoite protein (CSP) from the malaria parasite [25, 26]. Similar approach can be used to develop malaria vaccine using E71 octapeptide (SNTFINNA), which is an epitope from MSA2 derived from one of the two invariable domains of merozoite surface antigen (MSA2) of the malarial parasite *Plasmodium falciparum*. Fusion of E71 to the CP at the N-terminal region produced fusion E71-CP, which assembled to form chimeric PVLPs of JGMV when expressed in *E. coli* and baculovirus-insect cell. When administered to mice as a diphtheria toxoid conjugate in the presence of an adjuvant, it elicited a good anti-MSA2 antibody response and substantial protection to mice challenged with a lethal inoculum of rodent malarial species, *Plasmodium chabaudi* [18, 27]. Similarly, expression of a construct containing residues 37–248 of MSA2 also yielded MSA2-CP for the assembly of chimeric PVLPs. Another important parasitic disease is caused by *Schistosoma japonicum*, which is the human blood fluke parasite causing oriental schistosomiasis in a wide range of hosts, including carnivores, rodents, insectivores, and humans. Sj26-glutathione S-transferase of 26 kDa antigen from *S. japonicum* is the molecular target for antischistosomal therapy. Fusion of Sj26 antigen to the N-terminal of JGMV CP produced fusion CP, which assembled to form PVLPs of JGMV in *E. coli* [18, 28]. Administration of Sj26-PVLPs to mice without adjuvant elicited antibody responses comparable to monomeric Sj26 administered with Freund's complete adjuvant [29].

Vaccines for Japanese encephalitis

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus responsible for the acute encephalitis in human. It is affecting a vast geographic area that includes India, China, Japan, and almost all of Southeast Asian countries and resulting in 10,000 mortality every year. The survivors of this disease suffer from a long-lasting serious neurological and psychiatric sequelae [30]. Protection against JEV is antibody-dependent, and neutralizing antibodies alone can impart protection. The envelope (E) protein of JEV contains virus-neutralizing epitopes. Four peptides (A_{373–399}, B_{386–399}, C_{151–163}, and D_{303–400}) from JEV E protein were

fused to JGMV-CP and expressed in *E. coli* bacterial system. JGMV-VLPs containing peptide A, a 27-amino acid peptide containing amino acids from 373 to 399 of JEV E protein, induced virus-neutralizing antibodies without the use of an adjuvant. Mice immunized with these VLPs showed significant protection against a lethal JEV challenge [30].

Vaccines for contraception

A method of safe and effective contraception/castration by immunological interference is more effective than conventional methods. This can be achieved by conjugating an appropriate antigen to a carrier molecule to make the immune system to raise neutralizing antibodies. The prime candidates for immunoneutralization include reproductive hormones, gametes and/or their associated components. One of the important sites for immunological intervention is to inhibit the production of gametes (sperm and egg), functions of gametes, or their outcome. One of the important sites for immunological intervention is to block fertilization potential of spermatozoa. YLP₁₂, a 12-mer (YLPVGGLR-RIGG) testes-specific peptide, which is expressed in the acrosomal region of spermatozoa and has a role in human sperm-zona pellucida (ZP) interaction, can be an important antigenic candidate for immunoneutralization. ZP3 (QAQI-HGPR) peptide of ZP glycoprotein of oocytes can also be a potential antigenic candidate for developing contraceptive vaccine [31, 32]. Immunization of mice with genetically engineered JGMV-VLPs encompassing gamete epitopes of zona (ZP3), spermatozoa-specific (YLP12) and YLP12-ZP3 peptide without any adjuvant generated specific antibodies and significantly curtailed fertility [33]. Similarly, pituitary gonadotrophin secretion, which is stimulated by luteinizing hormone releasing hormone (LHRH) from hypothalamus, controls reproduction in mammals. Selective neutralization of LHRH by a specific antibody would be a potential alternative to surgical castration. Active immunization of mice with a decapeptide (QHWSYGLRPG) of LHRH, at the N- or at both N- and C-terminal regions of CP yielding LHRH-PVLPs, was immunocastrated [18, 34, 35].

Papaya mosaic virus (PapMV)

Papaya mosaic virus (PapMV) is a member of the genus *Potexvirus* in the family *Alphaflexiviridae*. It is a flexible rod-shaped (500 nm long, 14 nm diameter) virus with a neutrally charged particle composed of 1400 subunits of CP assembled around a positive stranded ssRNA of 6656 nucleotides. The CP of PapMV is composed of 215 amino acids and has a molecular mass of 23 kDa [36, 37].

Vaccines for viral diseases

Hepatitis C virus (HCV)

PapMV nanoparticles provide an efficient vaccine platform, in which the peptide antigen is fused to the C-terminus of PapMV CP. This results in viral particles that present surface-exposed epitopes that trigger humoral or cytotoxic immune responses. Hepatitis C virus (HCV) is a bloodborne virus and causes chronic and acute hepatitis infection worldwide, and approximately 700,000 people die each year because of hepatitis C-related liver diseases (<http://www.who.int/mediacentre/factsheets/fs164/en/>). Leclerc and coworkers first reported that the critical effect of multimerization of plant virus-based vaccine platforms is immunogenicity. They engineered PapMV CP as carrier protein with a C-terminal fused HCV E2 glycoprotein epitope to self-assemble into multimeric form (PapMVCP-E2) VLPs as immunogenic targets. When C3H/HeJ mice were injected twice with the multimeric form of VLP vaccine it triggered a long-lasting humoral response against both the CP and the fused HCV E2 epitope with a balanced Th1/Th2 response with a prevalence of immunoglobulins (Igs) IgG1, IgG2a, and IgG2b antibodies, and the less pronounced production of IgG3 anti-epitope antibodies. Notably, the immunogenic properties of PapMVCP_{27–215}-E2, which is unable to self-assemble, are lost in its monomeric form [38].

Influenza virus

Influenza is caused by a virus that attacks the upper respiratory tract and lungs. It is a seasonal epidemic and globally causes between 250,000 and 500,000 deaths annually (<http://www.who.int/mediacentre/factsheets/2003/fs211/en/>). PapMV CP carrying the universal extracellular domain of matrix 2 (M2e) influenza epitope (PapMV-CP-M2e) was developed as a universal influenza A vaccine. PapMV-CP-M2e triggered protective humoral response against a lethal influenza infection in mice. Furthermore, PapMV-CP VLPs were also found to act as an adjuvant that increased the immunogenicity of chimeric viral particles (CVPs) [39]. PapMV VLPs have also been an attractive platform for triggering cellular responses for cancer immunotherapy and vaccine development. Leclerc et al. [40] established that PapMV-CP-based VLPs can induce major histocompatibility complex (MHC) class I cross-presentation of HLA-A*0201 epitopes from melanoma antigen gp100 and from influenza virus M1 matrix protein. Although the C-terminus of the PapMV CP is located on the surfaces of VLPs, which is used for the fusion of

antigenic epitopes, there is a possibility that these antigenic fusion proteins interfere with the self-assembly of viral particles. A model antigen, influenza HA11 peptide, was used to test eight different sites of fusion located at the C-terminus of PapMV CP and self-assembling abilities of these constructs to form nanoparticles. Only three sites were found to produce stable self-assembled VLPs, that is, the C-terminus and positions directly after amino acids 187, and 12 (near N-terminus) [41]. It was also reported that residue F13 of PapMV CP is critical for the self-assembling of subunits to form nanoparticles [42]. In a proof-of-concept demonstration, Leclerc and coworkers inserted the influenza nucleocapsid (NP_{147–155}) epitope (a 9-mer H-2Kd epitope flanked by five native residues of NP) just before F13 in the N-terminus of PapMV CP. This insertion did not interfere the self-assembly of nanoparticles and triggers a cytotoxic-T-lymphocyte (CTL) response in Balb/C mice [43].

Vaccines for bacterial diseases

New adjuvants and immunomodulators that activate cell-mediated immune responses are of considerable medical importance as treatments for many life-threatening infectious diseases. PapMV VLPs containing ssRNA non-specifically triggered strong innate immune stimulation in the lungs of a mouse model by recruiting neutrophils, monocytes/macrophages, and lymphocytes, and conferred protection against a lethal challenge with *Streptococcus pneumoniae* [44]. Thus, PapMV nanoparticles can non-specifically induce innate immunity in lungs during viral pandemics or biological warfare. Similarly, self-assembled PapMV CPs containing non-coding ssRNA constitute a novel type of TLR7 agonist with strong immunostimulatory properties. Furthermore, pretreatment with PapMV improved effector and memory CD8⁺ T-cell responses induced by bone marrow-derived dendritic cell (BMDC) vaccination and increased protection against the foodborne pathogen *Listeria monocytogenes* challenge. Thus, it seems a PapMV platform could be used to develop T-cell vaccines for infectious diseases [45].

Vaccines for cancer

Cancer is a global epidemic causing a large group of diseases in any part of the body. It is the cause of 13% of deaths worldwide [46]. PapMV has a tremendous potential as a vaccine platform to activate antitumor immune responses in an interferon (IFN)- α -dependent manner. When PapMV was injected subcutaneously into implanted poorly immunogenic B16-OVA melanoma cells in a murine model, tumor progression was significantly retarded due to the

downregulation of Ki67, an activation/proliferation marker in B16 cells. In addition, tumor immunogenicity was enhanced by the up-regulation of the surface expression of IFN- α -mediated major histocompatibility complex (MHC)-I. Thus, it appears that PapMV has an intrinsic ability to trigger the antitumor T-cell immune system, which can be harnessed for cancer immunotherapy [47].

Papaya ringspot virus (PRSV)

Papaya ringspot virus (PRSV) is a non-enveloped, flexuous, rod-shaped particle measuring 760–800 nm in length and 12 nm in diameter in the genus *Potexvirus*, family *Alphalexiviridae*. It is transmitted in a non-persistent manner by aphids [48]. PRSV infection is responsible for significant losses in papaya by causing mottling and distortion of leaves and ringspots on fruit. Guerrero-Rodriguez et al. [49] expressed PRSV VLPs in *E. coli* and chemically coupled them to green fluorescent protein (GFP) to evaluate their potential use as antigen carriers. Although bioconjugate instability was observed, PRSV VLPs enhanced immune response against the antigen GFP in BALB/c mice by significantly increasing anti-GFP IgG response, particularly, IgG1 class response. Similarly, PRSV-CP was analyzed for the presentation of 15 amino acid (aa) antigenic VP2 peptide epitope of animal virus, canine parvovirus (CPV) in *E. coli*. Recombinant PRSV-CPs containing CPV epitope at the C-terminus and at both N- and C-termini elicited higher specific antisera in immunized mice [50]. Dengue fever is the most important mosquito-transmitted viral disease worldwide. It is transmitted to humans by infected *Aedes mosquitoes*, and there is no commercially available vaccine to prevent dengue fever in humans [51]. Here, PRSV was employed for the transient expression of domain III of the DENV 2 E protein (D2EDIII), a promising subunit vaccine candidate against dengue fever, in *Cucurbita pepo* (zucchini) plants as a fused protein with PRSV P1 protein. Thus, PRSV could be used as a stable vector for the expression of heterologous proteins in zucchini plants [52].

Plum pox potyvirus (PPV)

Plum pox potyvirus (PPV), also known as sharka, belongs to the *Potyvirus* genus in the *Potyviridae* family of plant viruses, and affects stone fruit worldwide. It is commonly spread by aphids feeding on infected plants and then transferring PPV to uninfected plants [53]. The potyvirus genome consists of a ssRNA of ~10 kb with a viral protein genome-linked (VPg) at its 5' end and a poly(A) tail at its 3' end encapsidated by a single type of capsid protein (CP) subunit [54]. The N-terminal part of PPV CP was chosen as a site for the expression of foreign antigenic peptides, because it is exposed on the virion surface and is highly

immunogenic. Canine parvovirus (CPV) is a highly contagious viral disease affecting dividing cells in a dog's body specifically in the intestinal tract. It also attacks white blood cells (WBCs) and damages heart muscle causing cardiac problems. Different forms of the antigenic peptide (single and tandem repetition) of VP2 capsid protein of CPV were cloned into PPV-NATM1uI vector. These PPV-CPV and PPV-2CPV chimeric virus particles (CVPs) were found to be immunogenic and to induce high levels of antibodies that neutralized CPV in mice and rabbits [55, 56].

Potato virus X (PVX)

Potato virus X (PVX) is the type species of *Potexvirus* genus and infects many members of the family *Solanaceae*. PVX is a flexuous rod (13 nm diameter, 515 nm long), monopartite, positive-sense, ssRNA plant virus, comprised of 1270 identical coat protein (25 kDa) subunits [57]. The crystal structure of PVX is unknown, but its architecture is predictable, because all *Potexviruses* share a common architecture with slightly less than nine protein subunits per helical turn [58]. High-resolution fiber diffraction studies of PVX have revealed helical symmetry with 8.9 protein subunits per turn and a helical pitch of 34.5 Å [59, 60]. Plant virus-based vectors can be exploited to produce genetically modified PVX for expressing a foreign protein fused to its CP, which self-assembles into CVPs. Green fluorescent protein (GFP) was fused to the surface-exposed N-termini of PVX CP, but because the sizes of GFP and PVX CP are similar, this fusion prevented virion formation due to steric effects. However, the assembly of PVX.GFP-CP virions was facilitated by the presence of free CP in addition to fusion protein subunits. Using a strategy based on FMDV 2A catalytic peptide, free and fusion proteins of PVX CP were generated by fusing the 2A sequence (16-amino acids) between the GFP gene and the N-terminus of the CP gene. This approach also allowed the expression of longer peptides or whole proteins [61].

Human immunodeficiency virus (HIV) is a bloodborne virus causing acquired immune deficiency syndrome (AIDS) by attacking the immune system. HIV-1 is the most common type and is found worldwide. Currently, no vaccine is available to prevent or treat HIV infection. Using genetic engineering, a highly conserved linear ELDKWA hexapeptide neutralizing epitope from glycoprotein 41 (gp41) of the ectodomain of HIV-1 was highly expressed as a N-terminal fusion protein with PVX CP. Mice immunized with the resulting PVX-derived CVPs produced high levels of HIV-1-specific immunoglobulin G (IgG) and IgA antibodies, demonstrating these CVPs offer the possibility of an effective vaccine against HIV [62].

Potato virus Y (PVY)

Potato virus Y (PVY) also belongs to the genus *Potyvirus*, and it is one of the most important helical plant viruses affecting potato production. Virions are flexuous filaments with 680–900 nm long and 11–13 nm wide. It contains single, linear positive sense ssRNA, about 9.7 kb in size. Each single coat protein contains 267 aa with 30–47 kDa in size. The viruses are transmitted by aphids in a non-persistent manner and some isolates are inefficiently transmitted by aphids and some are seed transmitted [63].

Vaccines for viral diseases

Hepatitis B virus (HBV)

Hepatitis B virus (HBV) infection affects nearly 300 million people worldwide, causing hepatitis B and risking persistent chronic carriers to develop hepatocellular carcinoma. The HBV envelope contains three surface glycoproteins, namely, the large (L), middle (M), and small (S) proteins, which is encoded by a single open reading frame that is divided into the preS1, preS2, and S regions [64–66]. preS1 (aa 20–41) was chosen as a model epitope to access the immunological properties to elicit virus-neutralizing and protective antibodies. The PVY-CP with an N-terminal insertion of preS1 retains the ability to form filamentous particles in *E. coli*. Mice that are immunized with these chimeric PVY-CP-preS1 derived VLPs exhibited a strong anti-preS1 immune response, even in the absence of adjuvants [67].

Vaccine for neurodegenerative disease

Alzheimer disease (AD) is a chronic neurodegenerative disease, which causes dementia due to the cerebral deposition of amyloid beta (A β)-peptide in the brain to form structures called 'plaques' and 'tangles.' This leads to the loss of connection between nerve cells, and eventually to the death of nerve cells and loss of brain tissue [68]. To circumvent this problem, active vaccination with amyloid β (A β) protein or passive immunization with anti-A β antibodies can be beneficial. However, long-standing presence of anti-A β antibodies or antibodies to immunogens homologous to the A β protein may produce protective effects. The amino acid sequence of the potato virus Y (PVY) nuclear inclusion b protein is highly homologous to the immunogenic N-terminal region of A β . PVY develop antibodies (anti-PVY antibodies) that can bind to A β within the Phe⁴-Ser⁸ and His¹³-Leu¹⁷ regions in both neuritic plaques and neurofibrillary tangles. Immune responses confirmed that exposure to PVY induced

antibodies that could influence the normal physiological processing of the protein and the development or progression of AD [69].

Tobacco etch virus (TEV)

Tobacco etch virus (TEV) is also a member of the genus *Potyvirus*. TEV infects tomatoes and peppers along with other plants in the *Solanaceae* family. The infected plant leaves show mottling, crinkling, distortion and downward curling, and plants infected at an early stage have shortened internodes and are severely stunted. The green peach aphid (*Myzus persicae*) and several other aphid species transmit the virus. The virus is also transmitted mechanically and by grafting, but not by seeds [63]. Porcine reproductive and respiratory syndrome virus (PRRSV) causes porcine reproductive and respiratory syndrome (PRRS), also named blue ear disease, is a widespread disease affecting domestic pigs. The symptoms include reproductive failure, pneumonia, and increased susceptibility to secondary bacterial infections. PRRS has been most economically significant swine diseases worldwide for over two decades [70]. PRRSV structural proteins include minor envelope proteins (GP2a, GP3, GP4, E, and ORF5a), major envelope proteins (GP5 and M), and the nucleocapsid protein (N). Self-assembled his-tagged TEV-CP-VLPs were evaluated for its adjuvant property. Up on subcutaneous immunization of mice with these TEV-CP-VLPs along with PRRSV antigenic chimeric protein (PRRSV_{chim}), which comprises PRRSV GP3 and GP4 epitopes, GP5 and M ectodomains and thioredoxin as fusion partner, elicited broader IgG2-specific antibody response against PRRSV_{chim}, when compared to the potent IgG1 response induced by PRRSV_{chim} alone [71].

Tobacco mosaic virus (TMV)

Tobacco mosaic virus (TMV) is a rod-shaped virus with helical symmetry that infects tobacco and *Solanaceae* family members. TMV is the type member of the genus *Tobamovirus* in the family *Virgaviridae*. It consists of a capsid containing 2130 identical coat protein subunits of 17.5 kDa each (159 amino acids) arranged as a right-handed helical rod (300 × 18 nm in size) with 16.3 subunits per turn around (+)ssRNA that forms a hollow protein tube with a central cavity of diameter 4 nm. The internal and external surfaces of its capsid protein consist of repeated patterns of charged amino acids, such as glutamate, aspartate, arginine, and lysine [72] (Fig. 2). TMV can undergo thermal transition to form RNA-free densely packed spherical nanoparticles (SNPs) of size 152 ± 58 nm [73, 74].

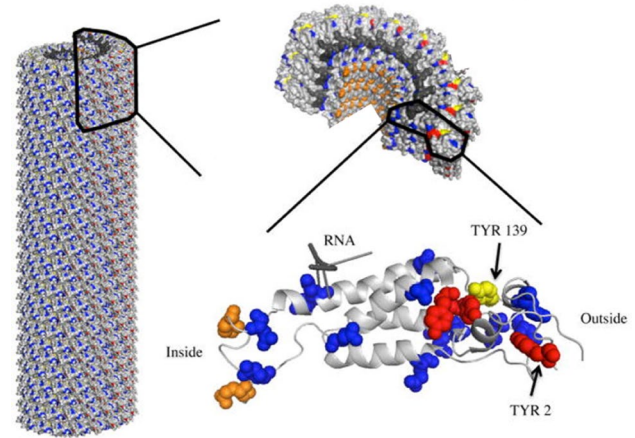


Fig. 2 A PyMol image of tobacco mosaic virus (TMV) showing highlighted exterior amino acids, that is, [Tyr139 (yellow) other tyrosine residues (red)], and glutamic and aspartic acid residues (blue) used for genetic modification and the bioconjugation of immunogenic peptides/proteins. Reproduced with permission from [74]

Vaccines for viral diseases

Human immunodeficiency virus (HIV)

TMV is an excellent immunogen and its antigenic properties have been extensively studied for several decades. Viral epitopes are categorized as cryptotopes, neotopes, metatopes, and neutralizing epitopes. Cryptotopes are hidden epitopes located deep inside the assembled virus particle and become accessible to antibodies after dissociation. Neotopes are specific to the quaternary structure of a virus particle and arise through conformational changes in monomers or through the juxtaposition of the amino acid residues from neighboring subunits. Metatopes are present in both polymerized and dissociated viral proteins, and neutralizing epitopes are recognized by antibodies and neutralize viral infectivity [75, 76]. HIV-1 Tat protein is considered as an important viral component of potential HIV vaccines. TMV-based transient expression system was used to produce edible HIV-1 Tat protein in spinach leaves, and feeding mice with Tat-producing leaves primed them for Tat antibody production and subsequent immunization resulted in significantly higher anti-Tat antibody levels compared to control mice [77]. TMV vector was also used to construct TMV particles with antigen presenting surfaces possessing three different epitopes, that is, two epitopes from the influenza virus hemagglutinin (HA) and one epitope from HIV-1 envelope protein gp120, in tobacco plants. Each of these TMV particles reacted with each anti-peptide antiserum [78].

Papillomaviruses

VLPs of plant viruses present antigenic epitopes to the immune system in highly ordered, repetitive, and quasicrystalline arrays that enhance immunogenic responses in the host system than free protein antigens. The only hurdle associated with the use of TMV as a vaccine scaffold is the limited number of amino acids that can be fused to its CP. Engineered rTMV with reactive lysine at its externally located N-terminus (GKGAG) facilitated biotinylation of the capsid for the conjugation of streptavidin fusion to either green fluorescent protein (GFP) or to the N-terminal fragment (61–71 amino acid) of canine oral papilloma virus L2 protein (COPV L2_{61–71}) (COPV causes warts in dogs) [79]. L2 is a papillomavirus minor capsid protein and a poor immunogen that requires potent adjuvants for antibody induction. However, unlike L1 (papillomavirus major capsid protein), L2 is a promising vaccine candidate as it induces antibodies that can cross-neutralize different papillomavirus species [79], and epitopes derived from the N-terminal proximal region of L2 can exhibit immunogenicity and cross-neutralizing immune responses. A peptide epitope in the region of 94–122 from the overlapping amino acid sequences of cottontail rabbit papillomavirus (CRPV) and rabbit oral papillomavirus (ROPV) was also tested for its effectiveness against these distantly related cutaneous and mucosal tissue-tropic papillomaviruses [80]. Recombinant TMV (rTMV) PVNs containing the 94–122 amino acid sequence in L2 were found to provide full protection against homologous papillomavirus infections and cross-protective immunity against distant papillomaviruses [81], and this was the first report of L2 peptide-based vaccination-induced cross-neutralizing immunity between two distinct papillomavirus types in an animal challenge model. Similarly, the TMV vector system has been used to produce vaccines against human papillomavirus 16 (HPV16) [82], and other diseases, such as HPV-8 [83], hepatitis C virus (HCV) [84], dengue virus [85], and lymphoma [86, 87].

Murine hepatitis virus (MHV) and rabies virus (RV)

Murine hepatitis virus (MHV) causes a variety of acute and chronic diseases in its natural hosts. In mice, MHV strain JHM usually induces fatal demyelinating encephalomyelitis and chronic demyelination diseases. MHV contains three structural proteins, and of these, spike glycoprotein (S protein) is a critical determinant of viral pathogenicity, and also contains major immunodominant neutralization domains [88]. TMV hybrids were constructed by inserting peptides 5B19 (L⁹⁰⁰LGICIGSTCA⁹⁰⁹) or 5B19L (P⁸⁹⁹LLGCIGSTCAEDGN⁹¹³) containing 5B19 epitope from MHV strain JHM S2 glycoprotein between amino acid residues Ser-154 and Gly-155 of TMV CP to result in

TMV-5B19 and TMV-5B19L, respectively. Mice immunized with hybrid viruses subcutaneously or intranasally survived challenge with a lethal dose ($10 \times LD_{50}$) of MHV strain JHM [89]. Bendahmane et al. [90] successfully developed TMV chimeras containing immunogenic epitopes 5B19 from MHV S-glycoprotein and G5-24 from rabies virus (RV) glycoprotein, and also demonstrated epitope charge and isoelectric point (pI) of the epitope displayed on the surface of TMV impacted its host interactions. The pI of CP fusion protein influenced the interaction between TMV and the host, and TMV CP tolerated only negatively charged peptides. Furthermore, CVPs comprised TMV CP fusion protein with isoelectric point of the peptide (pI):charge value closer to that of wild-type TMV CP caused infections in tobacco plants and protoplasts without causing cell death.

Poliovirus and feline parvovirus

Poliomyelitis is caused by poliovirus and is a highly infectious viral disease that paralyzes young children. The disease remains incurable but can be effectively prevented by immunization (<http://www.who.int/topics/poliomyelitis/en/>). A TMV-derived VLP-immunogenic epitope-based vaccine was first reported by Haynes et al. [91], who developed genetically engineered TMV VLPs expressed in *E. coli* that displayed VP1 peptide of poliovirus 3 (TMVCP-polio 3). Feline parvovirus causes fatal diseases, such as feline infectious enteritis and feline panleukopenia in cats, especially kittens, but immunogenic epitope sequences fused to recombinant plant viral structural proteins can be used as vaccines against these diseases. Genetically engineered antigenic fusion proteins were produced in tobacco using tobamovirus vectors containing feline parvovirus epitopes fused to the N-terminus of TMV CP, and these fusion proteins were used as vaccine antigens to induce protective immune responses against parvovirus. This methodology provides a safe, inexpensive means of producing vaccines against pathogens [92].

Foot-and-mouth disease virus (FMDV)

Foot-and-mouth disease (FMD) is caused by foot-and-mouth disease virus (FMDV), a prototypical member of the genus *Aphthovirus* of the family *Picornaviridae*. FMDV primarily affects cloven-hooved animals, such as cattle, pigs, sheep, and goats. Immunogenic dominant epitopes of FMDV serotype O, namely F11 (P¹⁴²–A¹⁵²) and F14 (R²⁰⁰–L²¹³) containing 11 and 14 peptides, respectively, from FMDV structural protein VP1 were fused to TMV CP between amino acids S154 and G155 to produce the recombinant viruses TMVF11 and TMVF14. Systemic infection of tobacco with these viruses produced TMV CP with FMDV epitopes, which exhibited protective immunity in guinea pigs [93]. The limited capacity of typical TMV-based vector was

overcome by developing a new improved TMV-based vector by deleting six C-terminal amino acid residues from the CP subunit that expressed peptide F11 of VP1 in tobacco. This new vector exhibited similar protective activities against FMDV in guinea pigs and swine. In addition, the capacity of foreign peptide was improved by expressing peptide F25 containing two fused epitopes (F14 and F11) of FMDV VP1, which was not possible using the original vector in tobacco. Thus, the new TMV-based vector can express longer foreign peptides for a range of vaccines [94].

Vaccines for bacterial diseases

TMV can also serve as a suitable platform for the delivery of bacterial antigens. In a proof-of-concept study undertaken to show TMV can serve as a platform to deliver multiple protective antigens of *Francisella tularensis* (the causative agent of fatal human disease, tularemia). Banik et al. [95] conjugated three different antigenic epitopes, that is, OmpA-like protein (OmpA), chaperone protein DnaK, and lipoprotein Tul4 to TMV to produce a TMV-conjugate vaccine with multiple *F. tularensis* antigens. This vaccine was later found to induce strong humoral immune response and to protect mice against respiratory challenges with high doses of *F. tularensis*. *Pseudomonas aeruginosa* is an environmental bacterium, which causes mild disease in immune-competent individuals, but in immunocompromised individuals, it becomes opportunistic and may be life-threatening. In particular, in cystic fibrosis patients, *P. aeruginosa* colonizes airways and causes morbidity and mortality. The outer membrane (OM) protein F (porin) of *P. aeruginosa* has been shown to have vaccine efficacy in animal models of chronic pulmonary infections, burn injury, and systemic infection [96]. Furthermore, chimeric TMV containing a 9–14 mer peptide (TDAYNQKLSERRAN) of OM protein F of *P. aeruginosa* between amino acids Ser154 and Gly155 of TMV CP (TMV-9-14), successfully immunized a mouse model of chronic pulmonary infection against *P. aeruginosa* challenge [97].

Vaccines for protozoan diseases

Malaria is a life-threatening disease caused by protozoan parasites belonging to *Plasmodium* species, which are transmitted by infected female *Anopheles* mosquitoes. Currently, there are no licensed vaccines against malaria. However, a malaria vaccine known as RTS, S/AS01 against *P. falciparum* has been subjected to clinical trials in several African countries. In an attempt to develop a malaria vaccine using chimeric plant virus VLPs as immunogenic carriers, the TMV CP surface was engineered to present a selected

malarial B cell epitope (12 amino acids) from *Plasmodium* circumsporozoite protein (CSP) by inserting it into the surface loop region or fusing it to the C-terminus of TMV CP using the ‘leaky stop’ signal derived from the replicase protein reading frame. It was found that wild-type TMV CP and fusion protein co-assembled into VLPs at the predicted ratio of approximately 20:1, indicating recombinant TMV can be used for the scalable and cost-effective production of easily stored and administered subunit vaccines [98]. Immunogenic *P. yoelii* merozoite surface protein 4/5 (PyMSP4/5) was expressed in tobacco leaves using a deconstructed TMV-based magnICON® expression system. Oral administration of PyMSP4/5 leaves with a mucosal adjuvant-induced PyMSP4/5-specific antibodies in naive mice and in DNA vaccine primed mice [99].

Cancer vaccine and immunotherapy

Due to the non-infectious nature of plant viruses in humans, TMV has been used to develop several types of vaccines to induce humoral and cell-mediated immunity in animals against various pathologies [12]. TMV particles interact specifically with lymphocytes and dendritic cells (DCs), which then become activated and express high levels of CD86, and this leads to superior CTL induction and tumor protection in vitro. McCormick et al. [100] first described the functional cellular response induced by TMV plant virus vaccines. Notably, TMV-peptide vaccines induced cell-mediated immune responses with less than 1 µg of peptide. To determine whether TMV-based multi-peptide vaccines are capable of immune activation and protecting from tumor challenge in vivo, TMV capsid with two MHC/Kb restricted T-cell epitopes derived from chicken ovalbumin (Ova) peptide (SIINFEKL), as a foreign antigen, and murine melanoma-associated peptide, p15e (KSPWFHTTL), as a self-antigen target, were evaluated in both the EG.7-Ova and B16 melanoma models employing Kb C57BL/6 mice. Both T-cell epitope peptide antigens bound directly to MHC class I and stimulated the T-cell receptor (TCR)-mediated activation of CD8⁺ T-cells. Furthermore, the introduction of a surface reactive lysine at the N-terminus of TMV CP and the chemical conjugation of immunogenic epitopes to TMV using a reducible bond linker were found to stimulate IFN-γ-producing T-cells after vaccination. Later, McCormick and coworkers tested TMV-fused melanoma peptide CTL epitopes as self-antigen-derived epitopes, and subsequently suggested that co-delivery of an immune-stimulatory peptide or co-delivery of a second peptide antigen was necessary to improve immune activation to the level necessary to protect mice from tumor challenge [12, 101]. Carbohydrate-based anticancer vaccines can also contribute to the induction of effective immune response during cancer immunotherapy.

Generally, high levels of weakly immunogenic tumor-associated carbohydrate antigens (TACAs) are expressed on cancer cell surfaces. Yin et al. [102] found that the chemical conjugation of monomeric Tn (GalNAc- α -O-Ser/Thr) antigen to tyrosine 139 of TMV capsid elicited strong immune responses by inducing high titers of IgG antibodies. TMV is an excellent vaccine epitope scaffold because of the immunogenicity of its viral particles, stability, and high accumulation rates.

Trastuzumab is a drug currently used for cancer therapy. It is a recombinant humanized monoclonal antibody that binds to the extracellular domain of human epidermal growth factor receptor-2 (HER2/neu) and inhibits HER2 signaling in tumors [46, 68]. rTMV PVNs displaying 36-amino acid HER2/neu peptide, that binds to trastuzumab (trastuzumab-binding peptide (TBP)), fused with the CP sequence were expressed in tobacco leaves by the *Agrobacterium*-mediated co-delivery of binary vectors encoding TMV RNA and TMV CP-TBP (TBP was inserted in C-terminal of CP). In addition, fusion of amino acid substituted TBP (sTBP), which binds trastuzumab, via a flexible peptide linker to TMV CP improved the manufacturability of rTMV [103].

Vaccine for contraception

TMV can also be used as an antigen carrier and delivery vehicle for administration via parenteral and mucosal routes. Vaccination for birth control has many advantages over tedious family planning procedures. The potential target for immunocontraception is the ZP3 glycoprotein of the zona pellucida. Fitchen et al. [104] showed that modified TMV VLPs expressing a hybrid coat protein containing a 13 amino acid sequence of the murine zona pellucida ZP3 epitope on its surface (a model contraceptive vaccine), elicited an autoantibody response that recognized zona

pellucida in mouse ovaries when parenterally administered to *C57BL/6J* and *BALB/CBY* mice (Fig. 3).

Zucchini yellow mosaic virus (ZYMV)

Zucchini yellow mosaic virus (ZYMV) is also a member of genus *Potyvirus*, family *Potyviridae*. ZYMV is a helical, flexuous filament of about 750 nm long and 11 nm wide, containing a single messenger-polarity RNA molecule of about 10 kb, encapsidated by ~2000 subunits of CP [105–107]. Arazi and coworkers [107] have demonstrated that chimeric ZYMV-CP replacing the surface-exposed N-terminal domain (NT) with a foreign peptide still restored the capability of the virus to spread systemically. They also used attenuated engineered ZYMV potyvirus (AG) as a non-pathogenic viral vector for the stable expression of heterologous proteins, such as human interferon-alpha 2 (hIFN- α 2) in cucurbits and squash [108].

Conclusions and future perspectives

Recombinant helical plant virus-based nanoparticle (rPVN) technology offers considerable advantages in the field of vaccinology, as many existing pathogens are difficult to culture in vitro. Here, we reviewed how genetic engineering can be used to utilize the helical capsid shells of various plant viruses to develop epitope presentation systems for vaccines. This approach also increases the immunogenicities of poorly immunogenic antigens due to the intrinsic abilities of PVNs to induce humoral and/or cellular immunity. Chimeric PVNs functionalized with immunogenic surface antigens produced in prokaryotic and eukaryotic expression systems have shown effective and safe immune responses in initial clinical trials. Nevertheless, the in vivo toxicities and

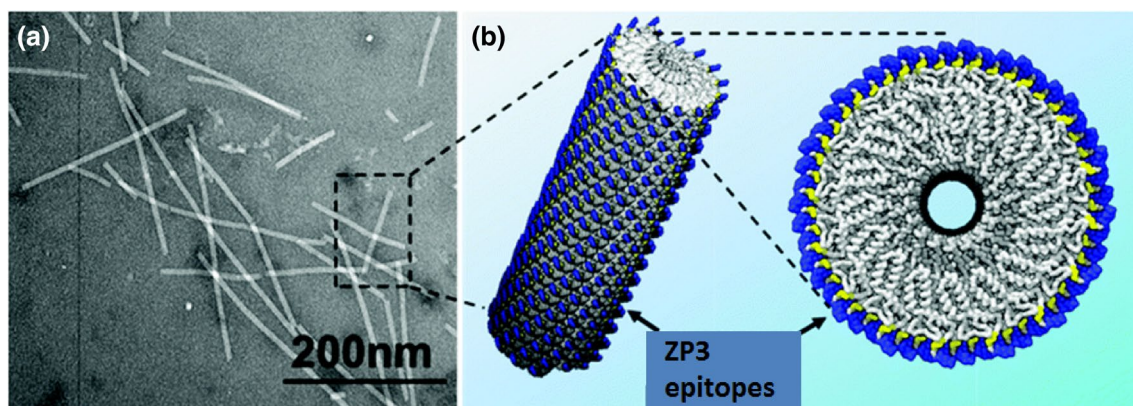


Fig. 3 **a** TEM image of purified recombinant TMV particles, and **b** recombinant TMV PVNs displaying the zona pellucida ZP3 epitope on their surface. Reproduced with permission from [6]

stabilities of these chimeric PVNs have yet to be evaluated. The successful display of multivalent arrays of immunogenic antigens on PVNs means different immunogenic epitopes from various pathogens can be combined and displayed on a single presentation system to achieve effective long-lasting immunity against different pathogens. Recombivax and Engerix-B are now available as VLP vaccines for hepatitis B, and a recombinant plant-derived malaria transmission blocking VLP vaccine has successfully completed a phase I clinical trial. Investigations are required to determine the size limitations, stabilities, and immunogenicities of different antigens at different positions on N- or C-terminals of coat proteins to optimize their presentations on the surfaces of helical PVNs.

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Compliance with Ethical Standards

Conflict of interest The authors have no conflict of interest to declare.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in this article.

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