Review Paper:

Plant Viruses: Versatile tools of Nanomedicine

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Abstract

Diseases due to pathogens such as viruses, bacteria, fungi or other parasites are fatal to both animals and plants. Plant viruses causes numerous diseases in plants and are mostly difficult to control due to vector-mediated transmission. Many fatal or deadly viruses some of which are animal-borne, with potential to efface human race, have surged up, the frequency of occurrence being high during the recent times such as Ebola, Swine flu, Nipah, Zikah etc., the latest addition being the pandemic COVID-19. Vaccination is based on the principle of cross protection and developing a new vaccine against a novel pathogen takes 12-18 months.

Virus expression systems or plant virus-based expression systems can be used to produce recombinant subunit vaccines. Repurposing of drugs is an alternative to combat fast spread of the pandemics. A potential but under-explored alternative is use of plant viruses or nanoparticles as transient expression systems for production of high yield of antigen or vaccine in plants and as nanocarriers of drugs.

Keywords: Cross protection, Molecular farming, Pandemic, Vaccine, Virus like particles, Recombinant Virus Nano Particles.

Introduction

"What Doesn't Kill You Makes You Stronger" Friedrich Nietzsche

Vaccination is a preventive or prophylactic and protective measure against pathogens and diseases. The basis of vaccination was illustrated and developed by Edward Jenner in 1796 against smallpox, a devastating disease caused by Variola virus. The material from a cowpox sore from a milkmaid's hand was inoculated into a healthy individual who never developed smallpox when exposed to the variola virus, months later.[9] Vaccination became widely accepted and gradually replaced the practice of variolation. Later, the virus used to make the smallpox vaccine changed from cowpox to vaccinia virus. The principle of vaccination was cross protection, where a pathogen provided immunity against another related pathogen or virus.

Cross protection is practised in plants as well, to control plant viral diseases. Cross-protection is a phenomenon in which infection of a plant with a mild virus or viroid strain protects it from disease, due to subsequent encounter with a severe strain of the same virus or viroid. It was first demonstrated in tobacco plants, where the plants already infected with a "green mosaic virus" (a TMV strain) developed no further symptoms when inoculated with a "yellow mosaic virus" (another TMV strain).[18,32]

Whenever a new disease or a novel pathogen emerges, vaccine and curative drugs have to be developed as the foremost weapon or defence against the pathogen. However, development of a new vaccine involves at least a year's time for testing and clinical trials for safety, immunogenicity and specificity. Recombinant vaccines are safer and more specific, but they are less immunogenic than traditional vaccines based on live or attenuated pathogens. Suitable adjuvants are required to achieve protective immunity. Plants and modified plant viruses have been explored as innovative platforms for production of vaccines and biopharmaceuticals, some of which are already approved and commercialised.[13]

Plant viruses have found a novel role as nanoparticles, not only as vaccines in prophylactic measures, protective tools against infectious or non-infectious diseases or metabolic disorders, to deliver drugs, for medical diagnosis, but also as sources of material for electronics and optics.[8] Plant-based vaccines are less expensive, give higher yield, are safe and easy to handle, store and transport at ambient temperature. Plant made vaccines thus are most suitable for immunisation programmes in resource-poor or developing countries. In this review, applications of plant viruses or plant virus-based nanoparticles in development of plant vaccines, biopharmaceuticals or medical therapeutics and diagnosis are discussed.

Development of Plant vaccine

Plant-based vaccines are low cost, requiring no external carbon source as they are fuelled by photosynthesis, are feasible and scalable, with possibility of large scale production, higher yield, are easy to prepare and administer requiring no sophisticated or sterile equipment or expertise for administration, are easy to store and transport requiring no cold storage facility, are safe since they are devoid of contamination with mammalian pathogens or toxic compounds and stable for years in seeds at ambient temperature. Most importantly, plants are eukaryotic systems where the expression, folding, assembly and glycosylation of the proteins are similar to that of the mammalian systems.[2,26]

Development of a plant vaccine involves selection or identification of the antigen of the pathogen causing the disease and the gene encoding it, designing and selection of a suitable expression or transformation vector, cloning the

gene for antigen epitope in the vector, transformation of the plant with the vector, regeneration and analysis of the transformed plants, isolation or purification of the antigen or immunogen if administered as vaccine (directly consumed in case of an edible vaccine), evaluation of the immunogenicity of the antigen protein through assays or tests and finally clinical trials in humans.[7]

Stable plant transformation using several techniques such as *Agrobacterium*-mediated method, direct transformation or gene delivery or biolistics, chemical or PEG-mediated transformation of protoplasts etc., integrates the transgene into the genome, so that the protein is expressed stably throughout various generations. The transgene can be targeted or integrated to nuclear genome or organelle genomes like chloroplasts. Higher yield of recombinant protein and maternal inheritance which prevents transgene escape through pollen are advantages of chloroplast transformation. The antigen protein can also be targeted to chloroplasts of cell wall or vacuole, for stable and higher yield, easy action or extraction.[11]

Generation of a transformed plant takes much time and coupled with the regulatory processes involved in genetically modified crops, the release of the product is delayed and the cost of production is increased. Also, these drawbacks drag down the potential technology and make it unsuitable for a pandemic situation, when the pathogen with a high fatality rate spreads in an alarming rate, such as that of the pandemic COVID-19 caused by novel corona virus SARS-CoV-2.

Plant cell suspension cultures and transient expression systems are viable alternatives for higher yield and rapid production of vaccines.[25] The tedious regeneration processes and somaclonal variations involved in *in vitro* regeneration techniques are overcome by these methods.

The plant virus-based expression systems are transient expression systems using whole plants, which can be either epitope presentation systems or polypeptide expression systems. The coat protein of the virus is fused with antigenic peptide or protein and displayed on the surface of coat protein after the assembly of the virus particle. Unfused but whole recombinant proteins are expressed in plants infected with the virus in case of polypeptide expression systems. Limited host range of the virus and limited size of the insert that can be used for production of functionally active antigen are constraints of plant-virus based expression systems.

Plant viruses as tools of therapeutics

Virus-based nanoparticles can effectively elicit an immune response and can be used for the targeted delivery for disease treatment and diagnostic purposes. Three types of virus-based nanoparticles can be used for eliciting immune response viz., Virus Like Particles (VLPs) and Virus Nano Particles (VNPs) and recombinant Plant-Virus Nanoparticles (rPVNs). VLPs are self-assembling coat proteins of viruses and lack genetic material. Hence VLPs are non-infectious and lack replication potential.

VLPs with replication potential in plants are called VNPs. Recombinant plant virus-based nanoparticles (rPVNs) are subtypes of VLPs which are non-infectious in mammals, but some retain their replication potential in plants. High immunogenicity even at very low doses in nanograms, is a major advantage of VLPs. First-generation viral vectors retain infectivity in the plants, raising safety concerns. Second-generation viral vectors or viral "deconstructed" vectors have minimum of viral elements required for replication of the vector and DNA delivery to the target plant which is *via* non-viral elements.[20,26]

Plant viruses are simple, rod shaped, or bearing icosahedral symmetry, having one or two repeated coat protein (CP) subunits and an RNA genome. Many plant viruses such as the tobacco mosaic virus (TMV), cowpea mosaic virus (CPMV), potato virus X (PVX), alfalfa mosaic virus (AlMV) and papaya mosaic virus (PapMV) are used for the development of vaccines. Non-enveloped 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), tobacco mosaic virus (TMV) and zucchini yellow mosaic virus (ZYMV) are ideal for preparing rPVNs.[20]

Recombinant Plant Virus Nanoparticles as vaccines, immunomodulators and adjuvants

rPVNs are less-expensive, amenable for storage at ambient temperatures, stable and yield higher levels of protein, making them suitable for inclusion in vaccination programmes of developing countries. Other low-cost systems such as bacteria and yeast produce insoluble proteins, thus restricting particle self-assembly. The baculovirus expression systems do not have these issues but are more expensive. The plant viruses are genetically engineered to express foreign antigenic epitopes from the pathogens.[21] The coat proteins (CP) of plant viruses are modified for the expression of fused antigens so as to display immunogenic epitopes on their surfaces. cDNA of the RNA virus genome along with the antigenic epitope is to be synthesised, to form the recombinant cDNA or rDNA encoding modified viral genome.

Normally, insertions of 10–50 amino acids are tolerated and expressed as closed loops on the surface of the virus. Insertions are made in the S-protein or N terminal region in case of icosahedral virus or N and C terminal in case of rod-shaped viruses, to form particles and for surface expression of antigens without destabilising the structure. To fuse larger peptides or complete proteins, the coat protein is biotinylated and streptavidin-conjugated peptides or proteins can be attached.

The cDNA can then be transcribed *in vitro*, to result in mRNA and can be inoculated into plants through systemic infection or through abrasion on leaves. Agroinfiltration is a widely used strategy where cDNA is inserted into a plasmid vector which is used to transform *Agrobacterium tumefaciens* and the recombinant *Agrobacterium* can be used to infect plants and induce transient expression where recombinant viruses are generated in the plants.[12]

The viruses can infect the plant, producing a systemic infection, generating multiple copies of the genome. The viruses can be purified from the plant and used for vaccine. Leaves can be harvested after few weeks post-infection followed by antigen purification. cDNA can be inserted into edible plants such as papaya, banana, lettuce, potato, carrot etc., which can be directly consumed as edible vaccine.

Tobacco mosaic virus (TMV)-based expression vectors are the most widely used vectors to produce foreign proteins in plants. A launch vector is developed with characteristics of *Agrobacterium* binary plasmid and plant virus expression vector. The CP gene of TMV is replaced by target gene or the gene encoding antigenic epitope driven by a viral sub-genomic mRNA promoter, is inserted between left and right border sequences (LB and RB) of T-DNA along with replicase and movement proteins, to form launch vector, which is transferred into plant cells by agroinfiltration. Multiple single-stranded DNA (ssDNA) copies of sequence between LB and RB are generated and released as virus particles.

Spherical nanoparticles devoid of RNA with ability to bind to different peptides or proteins were generated by thermal denaturation of the TMV CP or coat protein.[3]

Production in eukaryotic systems like plants allows post-transcriptional modifications ensuring that the rPVNs are similar to the parental virus and are more stable. rPVNs can induce humoral and cellular immunity or immune responses and are not infectious to humans or mammals. rPVNs also possess intrinsic adjuvant properties that can be used for immunomodulatory purposes. Infectivity of plant viruses to plants can be reduced or removed by inactivation of these rPVNs through chemical treatments or UV irradiation.

Some of the vaccines developed using plant virus nanoparticles are given in table 1. Papaya mosaic virus is an efficient adjuvant and vaccine platform in the design and improvement of innovative flu vaccines.[24] Papaya Mosaic Virus (PapMV) nanoparticles fused to the influenza CTL epitope triggered an immune response in both *in vitro* and *in vivo* models, but assembly of the recombinant PapMV CP into nanoparticles was crucial in triggering this efficacy which was linked to the stability of the nanoparticles at 37°C.[5] Some vaccines for malaria, anthrax, H1N1 flu, H5N1 flu etc., have completed the Phase 1 clinical trials in 2011-2015 itself.[13]

rPVNs also act as immunomodulators and adjuvants and are taken up by Antigen Presenting Cells or APCs, processed and presented to T cells. Dendritic cells were activated following TMV, PVX, CPMV and PapMV immunization.[12] Plant viruses have been shown to accumulate at solid tumors and elicit a highly localized immune response within the surrounding microenvironment.[8]

Plant viruses as nanocarriers of drugs

Plant viruses with highly ordered symmetry of icosahedral morphology can be used for molecular entrapment of drugs and have been engineered as nanoparticles. Empty virus-like particles of Cowpea mosaic virus (CPMV), Brome Mosaic virus (BMV), Red clover necrotic mosaic virus (RCMNV), Hibiscus chlorotic ringspot virus (HCRSV), Johnson grass chlorotic stripe mosaic virus (JgCSMV) and Physalis Mottle Virus (PhMV) can be used as nanocarriers of drugs and imaging chemicals. Drugs can be loaded through covalent attachment to certain reactive moieties on the capsid protein.

Interior cavity of a VLP can be loaded with drugs and the gated mechanism used, is sensitive to pH and metal ion concentration. VLPs swell and open pores at high pH, so that a drug cargo can enter and when the pH is lowered, the drug is trapped inside

Table 1
Vaccines developed using recombinant Plant Virus particles

Plant virus	**Vaccine/Disease**
Alfalfa Mosaic Virus (AlMV)	Respiratory Syncytial Virus[31]
Bamboo Mosaic Virus (BaMV)	Foot and Mouth Disease in Cattle[30]
Cowpea Mosaic Virus (CPMV)	HIV[6]
Cucumber Mosaic Virus (CMV)	Hepatitis C[22]
Papaya Mosaic Virus (PapMV)	Influenza virus and *Streptococcus pneumoniae*[17]
Potato Virus X (PVX)	Hepatitis C[29]
Tobacco Mosaic Virus (TMV)	Human Papilloma Virus (HPV)[16]
Tomato Bushy Stunt Virus (ToBSV)	HIV[10]

A variety of tissue-specific ligands can be conjugated to the exterior of the virus particle. Hibiscus chlorotic ringspot virus (HCRSV), could self-assemble into empty virus-like particles and used to transport drug molecules polystyrenesulfonic acid (PSA) and polyacrylic acid (PAA).[23] Folic acid was conjugated and used to encapsulate the anticancer drug doxorubicin, to elicit cytotoxicity in human ovarian cancer cells.[19] Red clover necrotic mosaic virus (RCNMV) was used as a drug delivery vehicle for doxorubicin. Plant virus nanoparticles were found to be more efficent than the PEGylated liposomal doxorubicin in the ovarian cancer model, but not in the melanoma cancer model.[14] Johnson grass chlorotic stripe mosaic viruses (JgCSMV) were also be used as nanocarriers for the anti-cancer drug doxorubicin.[1] VLPs derived from Physalis mottle virus (PhMV) are stable and robust and were functionalized to carry cancer drugs doxorubicin (DOX) and mitozantrone (MTX).[15]

Plant viruses for disease diagnostics

Virus nanoparticles can also be used for diagnostic purposes. The peptide or protein components on plant viruses or the aminoacid residues on the capsid protein can be linked chemically to dyes or polymers that can be used for detection or diagnosis of diseases. For example, Cowpea Mosaic Virus (CPMV) is an icosahedral nanoparticle with its capsid surface displaying 300 accessible lysine residues, each of which can be conjugated to various chemical moieties like fluorescent dyes or arrays, polyethylene glycol polymers and subcellular targeting molecules.[28] CPMV nanoparticles were thus constructed to display gastrin-releasing peptide receptors that are overexpressed in human prostate cancers.[28] The hybrid cowpea chlorotic mottle virus-based VLPs, stably assemble *in vitro* and package the RNA derived from sindbis virus, to protect against RNA degradation by cellular nucleases, so as to deliver the RNA within the cytoplasm of mammalian cells.

By fusion of subcellular targeting moieties, the hybrid VLPs could as well be targeted to distinct sites within the cell and used as drug delivery tool.[4] PhMV was conjugated to the fluorophore Cyt-5 to demonstrate that virus nanoparticles were efficiently internalized into breast, ovarian and prostate cancer cells.

Conclusion

PapMV as an adjuvant for the influenza vaccine and AlMV as a vaccine against malaria are few examples of the use of virus nanoparticles as immunomodulators and vaccines against diseases. Nanoparticles based on plant viruses are highly immunogenic but do not cause adverse reactions, though they are so far tested only on preclinical models.

Recently, Medicago Inc, a Canadian biopharmaceutical company has announced successful production of a Virus-Like Particle (VLP) as first step towards vaccine development against the novel coronavirus SARS-CoV-2, to be tested in preclinical trials for safety and efficacy. Plant viruses thus are potential tools to develop prophylactic and therapeutic nanovaccines, the weapons to fight against deadly, devastating, infectious diseases, epidemics and pandemics, as nano carriers of drugs and as diagnostic tools.

Future prospects depend on the efficient combination or synthesis of recombinant technology and nano technology to use the versatile, less-expensive platform of plant viruses as innovative, prophylactic tools against diseases for the benefit of mankind.

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