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Recent Advances in Virus Expression Vector Strategies for Vaccine Production in Plants

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Abstract

Plants offer tremendous advantages as cost-effective and safe platforms for the large-scale production of vaccines and other therapeutic proteins. Plant-derived vaccines represent a means by which to enhance vaccine coverage for children in developing countries, and can be administered orally to elicit a mucosal immune response. Plant-derived vaccines possess the dual advantage of preventing the antigen from degradation as it passes through the gastrointestinal tract, while at the same time being capable of delivering an antigen to the mucosal immune system. Plant virus vectors have been designed to express vaccine epitopes as well as full therapeutic proteins in plant tissue. This review describes recent advances with respect to plant virus expression vectors used as production platforms for biopharmaceutical proteins.

Introduction

The leading cause of infant mortality in developing countries persists from infectious diseases which are readily treatable in the industrialized world. One tactic used to combat this problem has been the establishment in 1992 of the Children's Vaccine Initiative, a platform by which globally accessible oral vaccines are generated through an assembly of philanthropic groups in conjunction with the World Health Organization. New vaccines developed under this initiative would be inexpensive, efficacious, safe, easy to transport to remote areas, and temperature stable [1,2]. The use of plants as production and delivery platforms for the expression of vaccine and therapeutic proteins is one promising approach that emerged from this Initiative. Biopharmaceutical proteins produced in plants retain the same structural integrity and activity as their mammalian-derived counterparts, contrary to bacterial expression systems. Vaccines produced from plants are protected from degradation by the harsh environment of the gastrointestinal tract via the plant tissues themselves, and can thus reach the mucosal immune system more effectively [3]. Vaccines and therapeutic proteins are now expressed stably in the form of transgenic plants, in a transient fashion by techniques such as agroinfiltration, or even by infection using recombinant virus expression vectors [4-6].

Gastrointestinal diseases such as infantile diarrhea remain significant causes of morbidity and mortality in many developing countries. Plant-made vaccines have the potential to enhance vaccine coverage in children and infants, particularly in resource-poor regions where such diseases remain a problem. Oral consumption of plant-based vaccines would be well suited for combating gastrointestinal diseases, and this possibility has been examined in the form of several Phase 1 clinical trials [7].

Many antigens do not become recognized by the gut as foreign and as a result cannot serve as immunogens. The use of adjuvants, which can affect the immunogenic context in which an antigen is encountered, is one way to overcome this problem. One of the most commonly used mucosal imunogens for plant-derived vaccine delivery is the Cholera Toxin subunit B (CT-B). Lacking toxicity to the cell, CT-B can not only modify the cellular environment in order to present the antigen in a highly efficient manner, but can also act as an efficient transmucosal carrier molecule and delivery system for plant-derived subunit vaccines [8]. In this instance, proteins which exhibit weak immunogenicity are coupled to CT-B and are then expressed in plant tissue. Proteins presented as a fusion protein of CT-B exhibit increased antigenicity within the gut [9].

A principal driving force for plant-derived biopharmaceuticals has been its expected potential to provide relief to Third World countries. Twenty percent of the world's infants remain unimmunized as a result of limitations on vaccine accessibility [10]. A number of infectious diseases such as dengue fever, hookworm and rabies are less renowned and treatments are poorly financed; developing vaccines in plant expression platforms could offer an opportunity to treat these 'orphan' diseases [10,11]. What makes plant virus expression vectors particularly attractive are the fact that they require no lengthy steps of plant transformation, yet they can express substantial levels of the gene of interest on a massive scale rapidly, often within a few days, by merely increasing the number of host plants. Moreover, vaccine proteins can be purified inexpensively from plants, or depending on the intended usage, may require only partial purification.

Besides providing vaccines and therapeutic proteins to those who reside in developing countries, plant virus expression vectors offer alternatives for other reasons as well. Production platforms based upon plant viruses have been used to produce vaccines against possible biological warfare agents, global pandemics and even certain cancers. Together, these applications provide compelling motivation towards further developing plant viruses as a technology to produce biopharmaceuticals and other proteins in plants.

A large number of mammalian therapeutic proteins are glycosylated, and plant proteins also undergo post-translational modifications; however, they are not identical to their mammalian counterparts. One end result of these subtle differences in glycosylation motifs between plant and mammalian derived therapeutic proteins could be an increase in allergenicity and other undesirable immune responses [12]. Today's plant glycobiologists have been able to further 'humanize' therapeutic proteins such as immunoglobulins by altering a variety of plant glycosylation pathways [13,14]. For example, transgenic 'knockout' plants have been generated which lack plant-

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specific glycosylases or other enzymes involved in plant-specific post-translational modifications. Other plants have been produced that express glycoproteins which are sialylated and O-glycosylated for correctly humanized protein expression [15]. Additional post-translational modifications, such as the inclusion of a signal peptide to retain proteins within the endoplasmic reticulum, can also be constructed in plants [16].

Plant-Derived Vaccines Can Engage the Mucosal Immune System

Sites of entry for infectious diseases are often the mucosa of the digestive, respiratory, and urogenital tracts, where antigen uptake, processing, and presentation for induction of mucosal responses take place. The Gut-Associated Lymphoid Tissue (GALT) represents a major portion of the body's entire immune system, whereas in the respiratory tract, antigens are taken up into alveolar spaces by antigen-presenting cells to regional lymph nodes [17]. B cells migrate via the lymphatic system to regional lymph nodes, where the primary immune response, including IgA and IgG antibody production, takes place [18].

Technology Platforms Used to Produce Vaccine Proteins in Plants

A variety of approaches can be employed to generate biopharmaceutical proteins in plants. The first available approach was to stably transform plant cells and regenerate them into mature transgenic plants. This is routinely accomplished by using either *Agrobacterium*-mediated transformation or biolistic delivery [19]. As an alternative to producing transgenic plants, which can be a lengthy process, plant viruses can be used to express foreign proteins [7]. Plant viruses have small genomes and are advantageous as they are relatively easy to manipulate. Infection of plants with recombinant plant viruses expressing the protein of interest can be an extremely rapid process, taking a few days to a couple of weeks to produce yields which are often greater than what is generally expected from stably transformed plants [20].

Transient Expression of Vaccine Proteins in Plants

Plant RNA virus expression vectors have developed further for use as effective vaccine and therapeutic protein production systems. Expression systems based on plant viruses include epitope presentation systems (in which a short antigenic peptide is fused to the virus capsid (coat) protein, and as a result is displayed on the surface of an assembled virion) and polypeptide expression systems (in which the entire foreign protein is expressed either alone or as part of a fusion protein) [7]. Geminiviruses, which are plant DNA viruses, accumulate to extremely high copy numbers in inoculated cells and thus have also been explored as potential protein expression platforms [21].

Among the most well known plant viruses which have been engineered to produce vaccines and therapeutic proteins include Tobacco mosaic virus, Tobacco rattle virus, Potato virus X, Cucumber mosaic virus, Cowpea mosaic virus and Alfalfa mosaic virus [7]. A wide variety of biopharmaceutical proteins have been expressed using full-length plant virus genomes [22-24]. These full-length virus vectors retain all of their genes necessary for infection; as a result, they can spread systemically throughout an entire plant. However, since these full-length, recombinant viruses can be transmitted from host plant to plant, biocontainment can be an issue for their use. Applications involving plant virus vectors can also be restricted as a result of insert size or narrow host range. To circumvent problems such as these, 'deconstructed' or modular versions of plant viruses have been under development for use as expression systems (Figure 1) [25,26]. Using this approach, deconstructed versions of the RNA viruses Tobacco Mosaic Virus (TMV), Potato Virus X (PVX), and Cowpea Mosaic Virus (CPMV) RNA-2 as well as the DNA geminiviruses Bean Yellow Dwarf Virus (BeYDV) and Beet Curly Top Virus (BCTV) have successfully been used been used to produce a variety of vaccine proteins in plants [27-29].

Phase 1 – Clinical Trials and Plant-Derived Vaccines

Tobacco mosaic virus

Tobacco Mosaic Virus (TMV), a single-stranded positive-sense RNA virus, is the most heavily utilized plant virus expression vector for biopharmaceutical protein production. TMV is a rod shaped virus that is encapsidated by over 2000 copies of coat protein; thus it represents an ideal epitope presentation system. The sizes of the epitopes which can be incorporated onto the virus particle surface are under strict limitations. Examples of some of the vaccine epitopes which have been expressed on TMV include antigens from papillomavirus, Foot and mouth disease virus, HIV-1, malaria and rotavirus [30-32]. Full-length proteins have also been expressed from TMV. Recombinant TMV viruses can express both their own coat protein as well as a protein of interest through the incorporation of an additional CP subgenomic promoter on the virus genome. Examples of full-length immunogenic proteins expressed from TMV-based vectors include L1 and E7 proteins of Human Papillomavirus (HPV), virus-like particles based on the coat protein of Norwalk virus, plasmodium antigen, α-trichosanthin, a tumor-derived ScFv, Bovine herpes virus-gD protein, major birch pollen antigen, and human growth hormone [25,30,33-37].

TMV has been used as a deconstructed expression vector with great success (Figure 1a). To this effect, the TMV genome has been 'deconstructed' into a series of separate modules, which contain the portions of the genome required for replication, as well as cassettes designed for insertion of foreign genes [26]. These recombinant virus vector modules can then be transfected into plants by a technology known as 'magnifection,' established by Icon Genetics. In this approach, an *A. tumefaciens* suspension is infiltrated into the intercellular space of all mature leaves of a tobacco plant, resulting in an infection that is more synchronous and rapid than systemic infection.

This innovative expression system using the deconstructed TMV replicon vector technique has been employed by many researchers. For example, Lindbo et al. [38], utilized a deconstructed TMV expression vector in conjunction with a viral RNA silencing suppressor to produce high levels of recombinant protein (between 600 and 1200 micrograms of recombinant protein per gram of infiltrated tissue) within a week post-infection. Another group produced a vaccine against the endemic disease plague, derived from the causative bacterial agent *Yersinia pestis*, using the deconstructed tobacco mosaic virus-based system [39]. Vaccine antigens purified from this system generated systemic immune responses and provided protection against an aerosol challenge by virulent *Y. pestis* in animal trials [40]. The research group of Saejung et al. [41] was also able to use a deconstructed tobacco mosaic virus-based transient expression system to express the envelope protein of Dengue virus. Mice immunized intramuscularly with Dengue envelope protein D2EIII exhibited neutralizing activity against Dengue type 2 virus. As another example, production levels of up to 300 mg/kg leaf fresh weight were determined for Hepatitis B virus surface Antigen (HBsAg) expressed in the TMV-based MagnICON™ viral vector expression system. These HBV vaccine antigens had the added

advantage of being able to self-assemble into virus-like particles [42]. Lastly, Webster et al. [37] expressed the malarial antigen PyMSP4/5 in *Nicotiana benthamiana* leaves using the same deconstructed TMV vector outlined above. Malarial antigen expressed reached levels up to 10% of total soluble protein or 1-2 mg/g of fresh weight produced. Moreover, the antigen retained its immunogenicity following long-term storage at ambient temperature within freeze-dried leaves. Mice that were fed this plant-derived malaria antigen along with a mucosal adjuvant, produced malaria-specific antibodies, supporting the concept that large quantities of malaria vaccine can be produced and stored using this TMV-based production system.

The deconstructed TMV expression system has also been employed as a potential treatment for various forms of cancer, such as Non-Hodgkins Lymphoma (NHL). NHL is currently the fifth highest cause of death in North America, and involves the uncontrollable proliferation of degenerate B-cells, which accumulate in the lymph nodes, bone marrow and other tissues. Degenerate B-cells of each individual NHL patient express a unique idiotype, which can be rapidly and inexpensively expressed in plants using a TMV-based virus expression vector agroinfiltrated into tobacco leaves. Using this strategy, adequate amounts of lymphoma vaccine can be rapidly and inexpensively produced for each patient. Plant-made vaccines expressed in this fashion offer a powerful short-term therapy to keep tumors in check [43].

A novel launch vector, constructed by Musiychuk et al. [44] and composed of TMV-based expression vector sequences harboured on the *Agrobacterium* binary vector pBI121 has been utilized for vaccine production. Using this expression system, multiple ssDNA copies of target sequences are released upon introduction into plant tissue. One hundred milligram quantities of protein per kilogram of plant tissue have been generated in less than a week using this expression system. Vaccines produced using the launch vector system include the oncogenic E7 protein of HPV, the H5N1 influenza virus HA and NA domains, and the F1 and V antigens of *Y. pestis*. These vaccines successfully protected against infection in animal models [45-47].

Cowpea mosaic virus

Among the other plant RNA viruses that have been investigated for their potential to produce biopharmaceutical proteins, Cowpea Mosaic Virus (CPMV) has been utilized extensively [28]. CPMV has a bipartite genome, replicates well in host plants, can readily incorporate vaccine epitopes onto exposed loops on the surface of its icosahedral virion, and can easily be extracted and purified from plants. Recombinant virus particles expressing foreign epitope sequences produce virus yields similar to that of wild type CPMV infection, and many of the epitopes which have been displayed on the surface of CPMV have been able to evoke strong immune responses [24]. Heterologous full-length proteins have also been expressed using CPMV as an expression vector system, such as the 2A protein of Foot and mouth disease virus. In this and other cases, the foreign protein has been expressed as a fusion along with the CPMV coat protein or movement protein, joined by an integral proteolytic cleavage site to allow the target protein to be released [48].

A disabled and replication incompetent version of RNA-2 of CPMV has recently been developed into a novel expression system

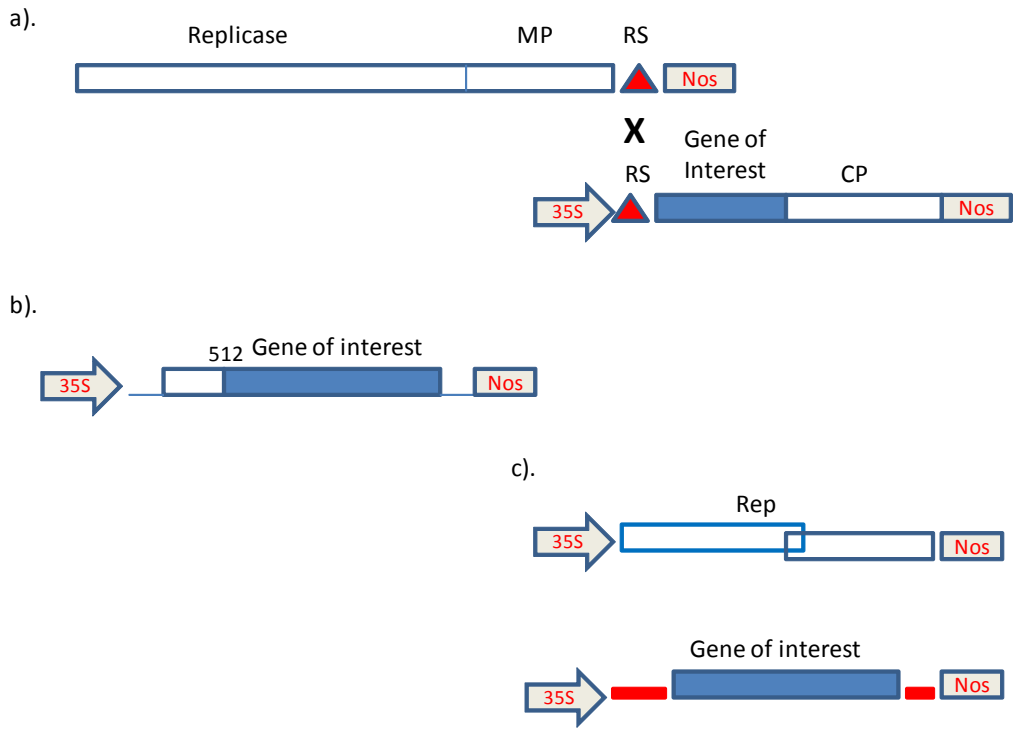

Figure 1: Schematic Representation of Deconstructed version of Plant Virus Expression Vectors. a). deconstructed TMV expression vector for production of active replicons in plants. VR: Virus Replicase, MP: Movement Protein, CP; Coat Protein, RS: Recombination Site. 35S: CaMV 35S promoter, Nos: Nopaline synthetase terminator.
b) CPMV RNA-2 nonreplicating construct for high expression of vaccine proteins. This is the RNA that is inoculated into plants for transient gene expression. NTR: Nontranslated Regions, 512: nucleotide position of CPMV RNA-2 first open reading frame, fused to gene of interest.
c) deconstructed BeYDV expression vector. Rep; Replication initiator protein, LIR: Long Intergenic Region, SIR: Short Intergenic Region.

(Figure 1b). Creation of this system involves the positioning of a gene of interest so that it lies between the 5' leader sequence and 3' Untranslated Region (UTR) of RNA-2. High level expression of a foreign gene is accomplished via *Agrobacterium*-mediated transient transformation; expression takes place in the absence of virus replication, a feature that makes this system extremely advantageous [49]. By removing an in-frame initiation codon located upstream of the main translation initiation site of RNA-2, researchers found that foreign gene expression could be increased dramatically. This system has been used to express at high levels proteins ranging in diversity from a full-size immunoglobulin to a self-assembling virus-like particle (10% and 20% of total extractable protein, respectively) [50,51].

Geminiviruses

More recently, geminiviruses have been developed for the production of plant-made biopharmaceuticals. Geminiviruses, named for their twinned capsid morphology, are small DNA viruses with broad host ranges, and can replicate to high copy numbers in infected cells [52]. Geminiviruses have been used in the past routinely to express foreign proteins in plants; and like TMV and CPMV, they have been developed further as expression vectors using the deconstructed strategy (Figure 1c). Bean Yellow Dwarf Virus (BeYDV), a member of the mastreviruses, has been designed so that its Replication initiator protein (Rep), required for virus replication, is under independent promoter control [53]. Using this approach, BeYDV–based expression vectors have produced a vaccine against Staphlococcus Enterotoxin B (SEB), considered to be a potential biowarfare agent, as well as Hepatitis B Virus, Norwalk Virus, HIV and HPV [53-55]. The BeYDV expression system has also been used to produce monoclonal antibodies against Ebola virus [55]. Another geminivirus known as Beet Curly Top Virus (BCTV) has been developed in a similar fashion for use as deconstructed vector for protein expression. Researchers working with this deconstructed virus further demonstrated that foreign protein expression could be enhanced further via co-delivery of an additional plasmid which expresses a viral suppressor of RNA silencing [56].

Plant-Made Vaccines, Allergies, and Oral Tolerance

Many foods and other substances that enter the gut are not highly immunogenic in general, and it is this actuality that prevents people from suffering from damaging inflammatory responses which may lead to conditions such as inflammatory bowel syndrome or food allergies. Alternatively, states such as oral tolerance are created by the reduction of succeeding responses to systemic challenge with a potentially immunogenic protein. It is imperative that plant-derived antigens be examined for their ability to induce oral tolerance to common allergens. Preliminary studies have been conducted on allergens derived from *Cryptomoeria japonica* (Japanese Cedar) as well as several asthma-based allergies [57,58]. Another concern is that plant-derived vaccines which are administered orally could bring about the development of tolerance to a particular vaccine, or even new allergies to co-administered food proteins. Is it possible that the accidental consumption of a plant-derived vaccine may lead to a later weak or ineffective response, and as a result, a reduced ability of the immune system to eliminate future infection? To address these concerns, threshold levels of orally administered plant-derived vaccines have been determined which did not stimulate detectable levels of antibody but nonetheless could induce immune priming. Studies related to the progression of oral tolerance or antigenicity via plant-derived biopharmaceuticals are currently being undertaken by various research groups [59].

Conclusions: Onwards and Upwards for Future Commercialization

Plant-derived vaccines can be produced in cell culture, open fields or in greenhouses. Since variations in soil and weather conditions can complicate outdoor production, cell suspension systems and greenhouses are preferred, as they offer more precisely controlled environments, a key step for obtaining Good Manufacturing Product standards. Plant cell line suspension cultures or hairy root systems which can secrete the specific protein product into the surrounding media are effective in reducing expensive downstream processing in an effective manner [11,60]. Fewer steps are required to purify vaccine proteins from plant tissues than standard purification from their mammalian and bacterial counterparts. A poultry vaccine for Newcastle disease, produced from a cell culture bioreactor system, was the first plant-derived vaccine to gain regulatory approval [61]. Several other plant-derived therapeutic proteins are currently completing the clinical trial phase and will soon approach market release. Many of these therapeutic proteins were produced from deconstructed plant virus expression vector systems. This review provides ample proof that plant virus expression vectors will provide a powerful tool for future production of biopharmaceutical proteins with a range of applications, including providing vaccines to the world's poor, combating future pandemics, and even to fighting cancer in the years to come.

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