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Review
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EXPERT REVIEWS

Development of plant-based mucosal vaccines against widespread infectious diseases

Expert Rev. Vaccines 9(8), 937–946 (2010)

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Mucosal vaccination is a perspective for the control of infectious diseases, since it is capable of inducing humoral and cell-mediated responses. In addition, the delivery of vaccines to mucosal surfaces makes immunization practice safe and acceptable, and eliminates needle-associated risks. Transgenic plants can be used as bioreactors for the production of mucosally delivered protective antigens. This technology shows great promise to simplify and decrease the cost of vaccine delivery. Herein, we review the development of mucosally administered vaccines expressed in transgenic plants. In particular, we evaluate the advantages and disadvantages of using plants for the production of mucosal vaccines against widespread infectious diseases such as HIV, hepatitis B and TB.

Keywords: hepatitis B • HIV • mucosal immunization • *Nicotiana* spp. • tomato • transgenic plant • tuberculosis • vaccine

According to the data of the WHO, during the last 30 years approximately 30% of the world's population survived without modern medicaments and appropriate medical service. Out of them, 47% of people are living in Africa, 65% in India, 29% in countries of the East Mediterranean and 26% in Southeast Asia. Countries with high income consume up to 90% of all medicamentous remedies produced in the world [1]. Many dangerous infectious diseases could be prevented with vaccination of infants under 1 year of age. Nevertheless, only 75% of children are provided with obligatory vaccines, while 27 million of children born annually remain practically without vaccination [2].

Today the production of vaccines is becoming more and more laborious and expensive, thus hindering global vaccination. In addition, a series of obscure questions still remain regarding prophylactic immunity. For instance, approximately 3 million newborns die annually regardless of whether they are vaccinated with parenteral vaccines [2].

Thanks to developments in the field of molecular biotechnology and immunology during the last 10–15 years, new strategies are now being considered for the production of new-generation vaccines. In this regard, one innovative approach is the use of mucosal vaccines.

The mucosa is the port of entry for many pathogens, in particular, bacteria and viruses that invade the host through respiratory, gastrointestinal or genital surfaces. The primary defense of these tissues is the mucosal immune system that can induce secretory IgA and serum IgG responses, thus providing two layers of defense against mucosal pathogens [3,4]. It has been estimated that the human mucosal surface sums up to 400 m^2 [5], and that mucosal-associated lymphoid tissue (MALT) contains 50% of total body immunity producing 70% of the body's immunoglobulin as secretory IgA [6].

Administration of vaccines onto mucosal surfaces is more effective than parenteral administration at stimulating a mucosal immune response and can also elicit humoral, cell-mediated and systemic immune responses [7]. Mucosal delivery has the additional advantage of not needing syringe administration, making immunization practice more acceptable and decreasing the cost of an immunization program. Needle-free vaccinations increase the speed of vaccine delivery and are safer for both the patients and the healthcare staff. It has been calculated that 3 million healthcare professionals are injured worldwide with needles infected with hepatitis B virus (HBV), hepatitis C virus (HCV) and HIV [8]. In addition, needle-free vaccinations eliminate the necessity of trained healthcare staff to deliver the vaccine [8]. Mucosal vaccines offer distinct advantages especially for *en masse* immunization under epidemic and pandemic threats as well as threats of bioterrorism. However,

10.1586/ERV.10.81

ISSN 1476-0584

traditional vaccines are usually administered intramuscularly and subcutaneously since injection delivers a known quantity of vaccine and results in the production of antibodies and lymphoid cells that are measured in blood samples without difficulty [9]. On the contrary, it is difficult to measure the dose of a mucosally delivered vaccine that actually enters the body [10]. In addition, the majority of vaccines are not immunogenic when delivered mucosally and need the use of strong adjuvants, such as the cholera toxin and the heat-labile enterotoxin of *Escherichia coli*, or effective delivery systems [11].

Two features make mucosal vaccines attractive prophylactic instruments: first, the generation of both mucosal and systemic immunity; second, the most specific and easy penetration of many pathogens is across the mucous membrane. For example, it was shown that dendritic cells (DCs) of mucosal surfaces specifically catch virions of HIV from inner cavities and facilitate their entrance into M cells of Peyer's patches as well as into inner submucous levels of lamina propria [12]. Therefore, the first line of defense can be raised at the initial step of interaction of pathogens with the mucosal membrane.

Despite their advantages, up to now few vaccines approved for human use are mucosally delivered; examples are oral vaccines against poliovirus, rotavirus, *Vibrio cholerae*, *Salmonella typhi* and a nasal influenza vaccine [9,13].

One promising strategy for the production of mucosally delivered vaccines is the expression of candidate vaccine antigens in transgenic plant tissues. Subunit vaccines produced in plants would be safe and inexpensive to produce, and some of these plant-produced vaccines, such as MucoRice, were stable at room temperature for 3 years [14]. In this article, we will evaluate the advantages and disadvantages of using transgenic plants for the development of mucosal vaccines. In particular, we will explore the potential of vaccines produced in plant cells against widespread infectious diseases such as HIV, hepatitis B and TB.

Mucosal delivery & immunity

The success of a mucosally delivered vaccine is based on activation of the mucosal immune system. This system is the primary defense of the mucous membrane lining the digestive, respiratory and urogenital tracts. These membrane surfaces are defended by a group of organized lymphoid tissue structures known commonly as MALT. MALT can be subdivided into sites such as the gut-associated lymphoid tissue (GALT), the nasopharynx-associated lymphoid tissue and the bronchi-associated lymphoid tissue [8]. In the intestinal GALT, mucosal inductive sites include the Peyer's patches, a large cluster of lymphoid follicles. The follicle-associated epithelium covering the Peyer's patches contains specialized epithelial M cells that transport intact macromolecules and microorganisms across the epithelial barriers directly to subepithelial DCs that then present antigen in adjacent mucosal T-cell areas [9]. M cells are flattened, epithelial cells that lack the microvilli that characterize the rest of the mucous epithelium. M cells also have a deep invagination, or pocket, in the basolateral plasma membrane that contains T and B lymphocytes and DCs [4]. Intact antigens and microorganisms are transcytosed into the pocket and than transferred to the antigen-processing and -presenting cells for the initiation of mucosal IgA responses [4]. Following antigen processing and presentation, IgA-committed B cells migrate to distant effector sites such as the lamina propria of the gut and respiratory tract. Dimeric IgA is secreted into secretions as secretory IgA that prevent the initial interaction of the pathogen with receptors on the mucosal cell surface [4]. Peyer's patches are also populated by B cells that produce serum IgG. Thus, local IgG synthesis can also occur in the mucosal tissues following mucosal vaccination [8,15].

Vaccine administered mucosally may be partly degraded before reaching effector sites on the surface of the GI tracts [10], therefore, either a large amount of vaccine needs to be administrated or the purified antigen should be protected from proteolysis through encapsulation [10]. Transgenic plants that express an antigenic protein are a promising strategy to produce and mucosally deliver large doses of protective antigens in encapsulated forms, as the plant cell walls protect the antigen from the acidic environment of the stomach and enable intact antigen to reach the GALT [3,7,10]. Plant-made mucosal vaccines have several advantages including their low cost of production, higher scale-up capacity, and easy storage and transport. Moreover, the production of plant-made mucosal vaccines could eliminate downstream processing of traditional vaccines such as purification, sterilization and refrigeration [16]. In addition, the production of vaccines in plants allows the introduction of post-translational modifications such as glycosylation, lipid modifications and disulfide bond formation [17]. Plant-based vaccines do not have shortcomings that are intrinsic of other expression systems. For example, plant RNA viruses, unlike animal, come through the gastroenteric tract without entering cells of the mucosa. There are no prions or other morbific pathogens dangerous for human in plants. In addition, the use of agricultural plants for the production of mucosal vaccines (i.e., fruits, vegetables, cereals and greengrocery plants) in the majority of cases reduces the possibility of allergy and autoimmune diseases. Mucosal vaccines based on fruits, leaves or seeds might be used as raw (uncooked) or dried material without the loss of immunogenic quality [18]. We have shown that transgenic tomato fruits containing antigenic proteins p24 (HIV-1) and HBV surface antigen (HBsAg) persistently inherit these proteins in seven examined generations in amount of 19–25 μg/g of total soluble protein (TSP) [19].

Mucosal delivery of several plant-produced antigens has elicited antigen-specific antibodies in mice and in humans. The list includes vaccines against cholera [20], rabies [21], Norwalk virus [22], diarrhea [23], hepatitis B [24] and others that are at the stage of preclinical and clinical trials on animals and humans [20]. Recent studies demonstrated that mice orally immunized with plant-derived vaccine generated both systemic and mucosal immune responses. By contrast, subcutaneously immunized mice developed only systemic immunity [25].

Plants used for the production of mucosal vaccines

Several expression systems can be used for the production of antigens in plants. Antigens can be expressed from transgenes stably integrated in the nuclear or plastid genome of transgenic plants, or from engineered plant viruses infected into the plant tissues [10]. The

decision is complicated by the fact that the plant production system can influence antigen content, stability, authentic conformation and cost of production [7]. Several plants have been used for the production of mucosal vaccines, including seed crops such as maize, soybean and rice. Expression and accumulation of antigens in seeds have several advantages such as high accumulation and stability of recombinant protein, and ease of purification. Recently, Nochi *et al.* expressed the cholera toxin B subunit (CTB) in rice seeds [25]. When mucosally fed, antigens from transgenic rice seeds were taken up by the M cells covering the Peyer's patches and induced CTB-specific serum IgG and mucosal IgA antibodies with neutralizing activity [25]. In addition, the rice-expressed CTB remained stable and maintained immunogenicity at room temperature for greater than 1.5 years and was protected from pepsin digestion *in vitro* [25]. Another possibility could be the use of transgenic single-cell cultures, such as the tobacco plant cell lines BY2 and NT1. These systems offer the advantages of high-level containment and the possibility of producing recombinant proteins in bioreactors so that good laboratory and manufacturing practices are easily applied. In addition, these lines have low alkaloid content so that they can be used for mucosal delivery [7,26]. Other plants used for the production of plant-based vaccines include tobacco, potato, lettuce, spinach, tomato, carrot, pea, alfalfa, soybean and other plants [18].

One challenge in mucosal vaccine design is that a 1000-fold higher level of vaccine needs to be administered mucosally compared with by injections [9,10]. However, up till now, the majority of plant-derived pharmaceuticals have been produced by nuclear transformation and stably integrated nuclear transgenes usually yield low levels of expression (0.01–0.4% of the TSP) since sites of integration into host DNA vary among transformed lines, expressed sequences can be subjected to gene silencing, and the foreign proteins are sometimes degraded in plant tissues [27,28]. A promising alternative for the expression of mucosal antigens is the production of recombinant proteins in transformed chloroplasts. Plastids have the potential to accumulate enormous amounts of recombinant proteins because of the high copy number (~10,000 copies) of the chloroplast genome in each plant cell. Oey *et al.* reported that a phage-derived lytic protein could be expressed to more than 70% of a plant's TSP [29]. Chloroplast transformation offers several advantages over nuclear transformation, including uniform transgene expression rates, no gene silencing and transgene containment. The chloroplast stroma also allows post-translational modification such as oligomerization and disulfide bond formation [28]. Plastid transformation combines characteristics of prokaryotic and eukaryotic expression systems and, therefore, can be a suitable platform for the expression of viral and bacterial antigens such as human papillomavirus (HPV)-16 L1 and CTB [16,30,31]. Another advantage of chloroplast transformation for the production of mucosal vaccines is that multigenic engineering is possible, providing the opportunity to coexpress antigens and adjuvants in plastids [16]. Since the first vaccine produced in transgenic plastids [30], a number of antigenic proteins have been expressed via chloroplast genetic engineering and several chloroplast-derived, mucosally delivered vaccines could induce an appropriate antigen-specific immune response and confer protection against pathogens [20]. Mucosal immunization with transplastomic plant material containing the plague fusion antigen F1-V without adjuvant conferred greater protection (88%) against a 50-fold lethal dose of aerosolized *Yersinia pestis* than subcutaneous immunization (33%) [25,32].

One disadvantage of using chloroplast transformation is that, up till now, the majority of the antigens produced in transplastomic plants have been produced in tobacco, which is not edible and unsuitable for oral delivery. However, recently the HIV antigens p24 and Nef have been expressed in plastids of tomato [33]. Although green tomatoes accumulated the HIV antigens to approximately 2.5% of the TSP, there was no expression in ripe red fruits [33]. The authors speculated that this was because ripe red tomatoes contain chromoplasts that are generally less active in plastid gene expression than chloroplasts.

Several therapeutic proteins and antigens against anthrax, cholera, malaria and autoantigen for diabetes were produced in lettuce chloroplasts [20]. Davoodi-Semiromi *et al.* reported the expression of CTB fused to two malarial vaccine antigens apical membrane antigen 1 (AMA1) and merozoite surface protein1 (MSP1) in tobacco and lettuce plastids [20,25]. CTB–AMA1 and CTB–MSP1 accumulated as up to 13.17% of the TSP in tobacco and up to 7.3 and 6.1% of the TSP in lettuce, correspondingly. Mucosal immunization of Balb/C mice orally with tobacco transplastomic leaves conferred 100% protection against cholera toxin challenge and inhibited proliferation of the malarial parasite [25].

Tomato as a production system

In the last 10 years, along with other systems, tomato plants have often been used for the production of plant-based vaccines and several investigators have considered tomatoes as a good option for biopharming [22,34,35]. Tomato are one of the most consumed vegetables in the world. Annually the consumption of tomato is estimated at the sum of US$15 billion. Tomato fruits can be consumed as raw, cooked material, in the form of pastes, juices and ketchups. One of the most famous compounds in tomatoes, lycopene, is not destroyed with drying or cooking. At the same time, tomato is an unpretentious plant and its growth is possible everywhere in the world, in open and close environments. Fruits can be consumed both fully matured and at greenish or yellowish ripening stages. Therefore, using tomato fruits as edible vaccines does not require antigen purification before vaccine delivery. In addition, tomatoes contain many natural, valuable compounds such as lycopene, which has antitumor activity [36], and natural steroid alkaloides (e.g., saponin esculeoside, α-tomatine and its derivatives dehydrotomatine and tomatidine that possess antimicrobial, fungicidal and anti-inflammatory features, and antitumor activity [37–39]).

It is of value that tomato contains the natural adjuvant α-tomatine [33]. O-β-D-xylopyranosyl-(1→3)-(O-β-D-glycopyranosyl-[1→2]-O-β-D-glycopyranosyl-[1→4]-O-β-D-glycopyranosyl-[1→4])-O-β-D-glycopyranosyl-tomatidine is a natural nontoxic adjuvant whose content can reach up to 0.5 mg/g of fresh weight in green fruits or, after drying, 1–34 mg/g of dry weight [37].

Tomato plants have high regeneration capacity. Isolated cotyledons of 10–14 days old seedlings have high organogenic and embryogenic activities. Placing explants onto murashide media

containing phytohormones (i.e., indole-3-acetic acid [IAA] and cytokinins) leads to callus formation followed by shoot regeneration and rhizogenesis.

Tomato seedlings can be genetically transformed using different techniques. They can be transformed by inoculation of explants with a suspension of agrobacterial cells and by agroinfection of cotyledons, apexes, leaves, ovaries and whole fruits. It is also possible to obtain regenerants using the flamingo bill methods [40].

Normal fruits with fully developed seeds were obtained after agroinfection of receptacle or ovaries that gave transgenic plants of T1 generation after selection on kanamycin 100 mg/l. In our work, green fruits infected directly with agrobacterial culture were transformed with the genes T- and B-cell epitope-containing immunogenes (*TBI*)–*HBS* (HIV-1) and *PreS2-S* (HBV). The presence of the transgenes in transgenic lines was detected by PCR, and Southern and northern blot analyses performed on DNA (or reverse transcriptase-PCR products) isolated from pericarp tissues. The presence of the antigenic proteins was analyzed by ELISA [41].

Zhang *et al.* compared the use of potato and tomato for the production of mucosal vaccines against Norwalk virus [22]. They came to the conclusion that tomato plants were more suitable for the creation of mucosal vaccines and that dried tomato fruits were more effective than dried potato tubers. According to these data, the tomato-based vaccine was ten times more immunogenic than the vaccine based on potato tubers due to the presence of tomatine. Zhang *et al.* speculated that the antigenic proteins were more stable in tissues of tomato fruits in comparison to potato tubers, probably owing to the presence of more effective inhibitors of proteases in tomato [22].

In some cases the use of the genes such as those from HIV [42], HBV [43], HPV [44], hepatitis E virus [45] and SARS [46] resulted in difficulties of growth and development of plants.

We considered that the production of transgenic plants could be improved if the gene *ugt* from corn, which encodes the synthesis of uridine–diphosphate–glucose-transferase, or by its trivial name, IAA–glucose synthase, was introduced [47]. The product of the expression of this gene catalyzes the binding of the phytohormone IAA into a conjugate form with glucose, thus providing a pool of bound IAA. Later, free IAA can be liberated and act as a growth activator in the proper tissue at the proper time [48]. In our work, after the introduction of the gene *ugt*, we received well developed tomato plants transformed with the gene *TBI–HBS* with abundant harvest up to four to five times [47].

Plant-produced mucosal vaccines against widespread infectious diseases

Trials to create mucosal plant-based vaccines against HIV were carried out at different times using the envelope gene *env* [49], transmembrane protein gp41 [50], genes of core proteins p17/p24 [51], as well as regulatory proteins of early events of replication Tat and Nef [52,53]. Antigenic dominants of these proteins synthesized in plants were able to induce immune responses in mice.

Scotti *et al.* investigated the possibility of expressing the protein Pr55GaG HIV-1, a protein that usually accumulates at low levels in nuclear-transformed plants, using plastid transformation [54]. In transplastomic plants the level of Pr55GaG expression increased up to 7–8% of the TSP after the fusion with RbcL, and the protein Pr55Gag formed virus-like particles (VLP) similar to VLPs from the baculovirus expression system.

Meyers *et al.* compared the expression of HIV-1 Pr55Gag, a truncated Gag (p17/p24) and the p24 capsid subunit using transgenic plants and transient expression via *Agrobacterium tumefaciens* and recombinant tobamovirus vectors, respectively [51]. Expression of Pr55Gag was low in all systems; however, the *Agrobacterium*-mediated transient expression of p24 and p17/p24 yielded more than 1 mg p24 per kg of fresh weight of tobacco leaves. In addition, chloroplast targeted protein levels were highest in transient and transgenic expression. The transiently expressed protein p17/p24 was not immunogenic in mice, however, it was able to boost both humoral and T-cell responses induced by the administration of the DNA vaccine pTHGaGC [51].

Lombardi *et al.* studied the possibility of using the protein negative regulatory factor (NEF) HIV-1, which participates in early stages of HIV infection, as a potential vaccine [55]. The accumulation of NEF is usually low in plants after stable nuclear transformation. NEF HIV-1 was expressed using agrobacterial coinfiltration together with well-known proteins with silencing suppressor activity (p25 potato virus X [PVX], p19AMCV and p19TBSV). p19AMCV was more effective as an inhibitor of silencing and its coinfiltration resulted in a threefold increase of NEF HIV-1 expression (up to 1.35% of the TSP).

In another study, the protein NEF fused with p24 HIV-1 accumulated up to 40% of the TSP in plastids of tobacco and tomato [33].

Production of binary candidate vaccine against HIV-1 & HBV simultaneously based on transgenic tomato

It should be mentioned that HIV weakens the human immune system and is usually accompanied by other diseases that can cause the death of the patient. For example, AIDS often appears as an associated infection with hepatitis B and TB. Therefore, it is rational to perform the prophylaxis against HIV together with the prophylaxis against HBV. Hence, we tried to create a binary vaccine simultaneously against HIV-1 and HBV based on the chimeric gene *TBI–HBS*.

It was shown that genes encoding proteins of HIV have homologous sites with approximately 15,000 human genes such as those encoding interferon receptors, receptors of macrophages, colony-stimulating factors, mast cells, growth factors of the eye lens, fibroblast growth factor receptors and many others [56]. Therefore, for the creation of the chimeric gene *TBI*, Eroshkin *et al.* used polyepitopic immunogenes containing fragments of viral proteins that were not found in human proteins (autoimmunogenic epitopes) [57]. There were five potential B-cell epitopes and four epitopes stimulating T-helper (Th)-cell responses in the artificial polyepitopic protein TBI. Six neutralizing epitopes were taken from the envelope protein Env (HIV) and three epitopes from the core protein encoded by the gene *gag*. The sequences of epitopes used were reflected in the name of the artificial gene *TBI* able to stimulate the synthesis of neutralizing antibodies. The sequence of

the main neutralizing epitope of the Env protein, IQRGPGRAF, was also introduced into the polyepitope TBI [57]. In addition, a sequence encoding 226 amino acids of HBsAg, the main antigenic protein of HBV envelope, was introduced in the synthetic gene *TBI–HBS*. As a result, the construct TBI–HBsAg encoded for HIV and HBV epitopes and the activation of immunity against associated infections with HIV and HBV was obtained.

During the years 2002–2009, we studied the expression of the synthetic gene *TBI–HBS* and *PreS2-S* in transgenic tomato plants, with the same team. In the frame of this work, fresh, lyophilized and incapsulated vaccines were obtained (Figure 1). Results of these investigations were described in a series of publications [19,58–63] in which it was demonstrated that the genes *TBI–HBS* and *PreS2-S* were able to induce the synthesis of antigenic proteins in tomato fruits that were used as candidate vaccines in animal trials. These trials have shown that the mucosal vaccine produced in transgenic tomato was able to induce in mice both mucosal and systemic responses after feeding with lyophilized fruits [59–62].

As mentioned previously, the synthesis of the antigens produced in transgenic tomato plants transformed with the gene *TBI–HBS* was stable for seven generations (and perhaps longer, but experiments were not done) [19].

In addition we investigated how long the antibodies raised against TBI–HBS were present in mice during their lives. We determined that during 9–11 months after vaccination the level of antibodies, measured as amount of anti-HBsAg, was high enough. There was a decrease in antibodies to the level of the control after 19 months. It should be mentioned that the life expectancy of mice is approximately 2 years. Thus, mice maintained the immune response practically half of their lives after vaccination with the tomato-based vaccine [63].

Mucosal vaccine against TB

Annually, TB causes nearly 2 million human deaths worldwide, mostly in developing countries, and currently almost 2 billion individuals are infected with *Mycobacterium tuberculosis* [13,64]. TB is also a leading cause of death among HIV-infected individuals, with coinfection accounting for up to a third of deaths associated with AIDS [64]. *Mycobacterium bovis* is the cause of animal TB that can affect animals in the wild or in captivity, and is responsible for approximately 6% of total deaths through zoonosis [64]. Most of the world's population is vaccinated with the only approved TB vaccine: bacillus Calmette–Guérin (BCG), an attenuated vaccine derived from *M. bovis*. BCG vaccination protects against childhood forms of TB, but efficacy in adults against pulmonary TB ranges from 0 to 80% in different trials [13,64]. The induction of a mucosal immune response is important for protection against diseases for which entry and pathogenesis are clearly related to the mucosal system. Since the mucosal surfaces of the lung are normally the organ in which TB infection is initiated and is the major site of pathology, an immune response in the lung could play a major role in restricting initial infection and colonization of *M. tuberculosis* and *M. bovis*. However, the parenteral route of immunization, which is normally used for vaccination with the BCG vaccine, does not elicit optimal immune responses in the lung.

Since mucosal delivery can induce a common mucosal immune response, mucosal immunity in the respiratory tract can be stimulated by oral administration of an appropriate antigen. Needle-free vaccine administration also has the advantages of being safer and of eliminating problem of infections transmitted through the repeated re-use of needles. This last point is particularly important considering the high rate of HIV–TB coinfection in developing countries [65].

The protein antigen 85 complex B (Ag85B) and early secreted antigen target 6 kDa (ESAT-6) are two of the major antigens produced by *M. tuberculosis* during infections and are both capable of inducing strong immune responses in a number of animal models [11]. Zelada *et al.* demonstrated the expression of the ESAT-6 protein in *Nicotiana tabacum* leaves using a vector based on PVX [66]. The ESAT-6 open reading frame was expressed as a fusion protein with the 2A catalytic peptide of foot-and-mouth disease virus and the amino terminal of the coat protein (CP) of PVX. The PVX-based vectors produced virions

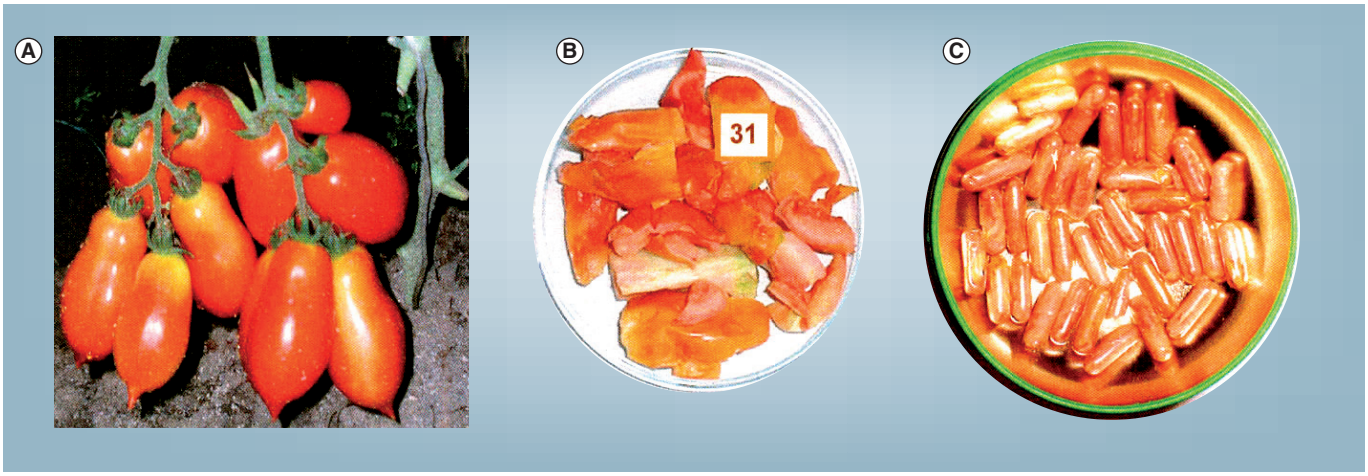

Figure 1. Tomato-based binary vaccine against HIV and hepatitis B. (A) Fresh vaccine, **(B)** lyophilized vaccine and **(C)** incapsulated vaccine.

with a chimeric capsid composed of the fusion ESAT-6–2A-CP and native PVX CP. Yields of ESAT-6–2A-CP fusion in agroinfiltrated tobacco leaves ranged from 0.5 to 1% of the TSP [11]. A system based on transient replication of plant virus vectors was also used by Dorokhov *et al.* [67] for the expression of the antigens Ag85B, ESAT-6 or Ag85B–ESAT-6 fusion in plant leaves. The technology used by Dorokhov *et al.* comprised the construction of tobacco mosaic virus-based vectors with the CP genes substituted by those for TB antigens [67], the delivery to *Nicotiana benthamiana* leaves of binary vectors containing a cDNA copy of the vector virus genome by agroinjection, and the coexpression of a protein suppressor of virus-induced gene silencing (P19) with the virus vectors [68]. This technology allowed the production of a very high level of the TB antigens in plants cells; particularly the level of Ag85B accumulation ranged up to approximately 800 mg/kg of fresh leaves [68].

Recently, Floss *et al.* reported the expression of the Ag85B and ESAT-6 antigens as an elastin-like peptide (ELP) fusion in transgenic tobacco plants [69]. Mice and piglets were injected with the fusion protein TBAg–ELP purified using inverse transition cycling or with crude tobacco leaf extract, respectively. Antibodies recognizing the TB antigens were produced in mice and piglets. In addition, in mice a T-cell immune response able to recognize the native TB antigens was detected [69].

Rigano *et al.* investigated the ability of transgenic *Arabidopsis thaliana* plants to produce a fusion protein consisting of the B subunit of the *Escherichia coli* heat-labile enterotoxin (LTB) and ESAT-6 for the development of a mucosally delivered and targeted TB vaccine [65]. Both components of the fusion protein retained native antigenicity and the ability to form pentamers. The levels of LTB–ESAT-6 production in plants varied between 11 and 24.5 µg/g fresh weight. A food-processing technique was used on transgenic tissues to standardize the antigen concentration in plant material and elongate shelf life. *Arabidopsis* material was pooled and freeze-dried. The pooling of processed *Arabidopsis* samples provided a batch of plant material with sevenfold concentrated, antigenically active fusion protein of uniform concentration [65]. To determine the ability of mucosal vaccination to generate immune responses, mice were fed with the freeze-dried transgenic *A. thaliana* material [68]. The plant-synthesized LTB/ESAT-6 fusion protein induced antigen-specific responses from $CD4^+$ cells and increased IFN-γ production and, therefore, induced a Th1 response in the mesenteric lymph nodes. In addition, mice fed the transgenic material generated a type 2 response in the Peyer's patch. Thus, the plant-derived TB antigen was delivered to the GALT and was able to prime an antigen-specific Th1 response [68].

Recently, Matvieieva *et al.* produced transgenic lettuce plants (*Lactuca sativa* L.) with genes coding the synthesis of the TB antigens ESAT-6 and Ag85B [70]. Lettuce was chosen because this plant grows quickly and does not require any thermal treatment before its consumption [70]. As an alternative, carrot transformation was used for the production of a mucosal vaccine. Ling-Jian *et al.* reported the expression of the *M. tuberculosis* MPT64 protein in transgenic carrots [71].

Expert commentary

Methods of increasing the immunogenicity of mucosally delivered vaccines

There are several issues regarding the mucosal delivery of subunit vaccines, such as degradation of the antigens by digestive enzymes, inefficient transport from the gut lumen to the GALT and induction of systemic immune tolerance [72]. In addition, mucosally administered subunit vaccines that consist of soluble, nonparticulate antigens have inherently low immunogenicity and can induce immune tolerance [9]. Mucosal vaccines can be more effective if they are multimeric and/or particulate. Viral structural proteins can self-assemble into organized macromolecular particulate structures called VLPs [72]. VLPs are promising mucosal vaccines because their size is appropriate for uptake by M cells and DCs, they can activate an innate immune response, and their structures mimic the form of an authentic virus [9]. Antigens of different origins forming VLP were produced in plants and were shown to assemble into VLPs [73]. Fernández-San Millán *et al.* reported the expression of the major structural protein of the HPV-16 capsid, L1, in tobacco chloroplasts [31]. In plants, a very high expression level of the protein L1 was achieved (24% of the TSP). The chloroplast-derived L1 protein self-assembled into VLPs that were highly immunogenic in mice after intraperitoneal injections inducing a specific humoral response [31]. Norwalk virus capsid protein was expressed as VLP in tobacco, potato and tomato fruits, and the VLP produced in potato and delivered orally stimulated serum IgG and IgA responses in humans [72].

Codelivery with an adjuvant or targeting protein can also increase the ability of the mucosal immune system to recognize plant-derived antigens. Two such carrier molecules for subunit vaccines are the heat-labile toxin of enterotoxigenic *E. coli* (LT) and the cholera toxin of *V. cholerae*, two toxins recognized as very potent mucosal adjuvants [7]. LT and cholera toxin consist of nontoxic B subunits and enzymatically active A subunits. Conjugation with LTB and CTB may facilitate antigen delivery and presentation to the GALT owing to their affinity to the GM1 gangliosides found on the surface of mucosal epithelial cells [7]. Several studies report the use of LTB and CTB as carrier proteins in plant-derived vaccines [7,68]. For instance, Zhang *et al.* recently produced transgenic rice seeds expressing the *Chlamydophila psittaci* antigen (MOMP) fused to LTB [74]. Oral immunization of mice with the rice material was able to induce mucosal and systemic immune responses [74].

Cholera toxin B subunit is also an efficient mucosal carrier molecule for the induction of oral tolerance to antigens and allergens [75]. Takagi *et al.* expressed in rice seeds T-cell epitopes derived from major Japanese cedar pollen allergens Cry j 1 and Cry j 2 fused with CTB or rice glutelin as a control [75]. Feeding mice with the transgenic material suppressed allergen-specific IgE responses and allergic symptoms at 50-fold lower doses of T-cell epitopes than required when using control seed [75].

The use of natural adjuvant can be very important in vaccine formulation. Morrow *et al.* demonstrated that the usage of tomatine highly increased the immune response against malaria, the pathogen *Francisella tularensis* and experimental tumors [39]. They showed that tomatine affected events occurring at the level of antigen-specific

receptors CD8, CD80 and CD86, increased the level of interferon and the activity of cytotoxic T lymphocytes, and blocked the infection of erythrocytes by *Plasmodium* malaria. It is obvious that the potential ability of tomatine should be studied urgently since it allows to combine soluble, subunit, plant-based vaccines with this accessible and inexpensive natural adjuvant.

Another challenge in the design of a mucosal vaccine is that it is impossible to determine exactly what dose of the vaccine actually crosses the mucosa, so a larger dose of vaccine is usually required. Future investigations should focus on the improvement of diagnostic methods of antigenic proteins, since a part of the antigens is inevitably lost in the GI tract. In addition, plants can modify a recombinant protein so that the immunoassay used cannot estimate correctly the quantity of the antigens. One well-known example is the production of lactoferrin in tobacco plant cells; in this case, lactoferrin was present in a modified form but its activity was increased up to 1000-times [76].

Downstream processing

To standardize and concentrate the vaccine in plant material it is possible to apply food-processing techniques to transgenic plant tissues. Freeze-drying is a simple and inexpensive technique that can provide antigen stability at ambient temperature. This technique has also been applied to tomato-made vaccines [62,72,77].

Zhang *et al.* expressed the recombinant Norwalk virus capsid protein in tomato and potato using a plant-optimized gene and tested the immunogenicity of dried tomato fruit and potato tuber fed to mice [22]. The authors demonstrated that recombinant Norwalk virus in tomato fruit is a more potent immunogen than potato. In the same paper, the authors demonstrated that air-dried tomato fruit stimulated a stronger immune response than freeze-dried fruit, perhaps because air-drying limits the destruction of plant cell matrix and membrane systems that can occur with freeze-drying [22].

In our experience, lyophilized material is the most acceptable method for vaccine delivery. It should be mentioned that green and red tomatoes could differ in the content of antigenic protein. For example, the content of antigenic protein HBsAg (HBV) was high in green and yellowish tomato fruits, but the level of HBsAg severely decreased in mature red fruits. In addition, the content of tomatine is up to 10,000-times higher in green tomato fruits than in red tomato fruits [37].

Another issue to consider regarding delivery approaches is the correlation between immune response and antigen purity as some plant cell components may interfere with the performance of recombinant antigens. In this regard, Portocarrero *et al.* examined the antibody response induced in mice immunized intramuscularly or intranasally with a plant-derived pB5 smallpox subunit vaccine administered with or without plant TSP [78]. The authors demonstrated that increasing the amount of TSP inhibited the pB5-specific response in the immunized mice and that intranasal administration was more sensitive to the presence of TSP contaminants than parenteral immunization. In addition, the recombinant smallpox vaccine administered intranasally required a larger dose of antigen to induce an antibody response comparable with that obtained by parenteral immunization. Finally, the plant-derived B5 administered to mucosal surfaces induced specific IgG and IgA responses, while intramuscular immunization produced only high serum IgG titers [78].

Based on the presented results, mucosal vaccines could be formulated as lyophilized, green or ripening tomato fruits. Another possibility could be the delivery of vaccines as lyophilized extracts prepared from freeze-dried tomato fruits. Freeze-dried tomato fruits can be stored for a long time without the access of moisture and air, and without any loss of antigenic proteins. In our work, there was no significant loss of antigenic proteins in samples of freeze-dried tomato fruits upon 2 years storage at room temperature [Salyaev RK & Rekoslavskaya NI, Unpublished Data].

Five-year view

The plant-made vaccine of the future has to have the same efficacy and safety of other pharmaceuticals produced by other sources [7]. For these reasons, it is likely that the future plant-produced vaccine product will be processed and purified plant material and will not be delivered in fresh form. Probably, the plant material will not need to be extensively purified but more likely simple and inexpensive food processing techniques, such as freeze-drying, will be applied [15,63,79]. For effective protection from proteases of the gastric juices, vaccine might be encapsulated by using gastric-soluble material.

Transgenic plants are very useful to produce and orally deliver vaccine antigens. However, technical problems, insufficient funding and the lack of commercial interest to conventional vaccines have slowed down the advancement of this technology [17,68]. Therefore, it will be of importance to continue the effective development of mucosal vaccines and, in particular, their clinical trials for widespread use in medical practice.

Another issue hindering the use of plant-made vaccines is the public confusion about genetically modified plants. In this regard, new methods to produce marker-free transgenic plants should be developed [33]. In this regard, it will be quite reasonable to not use marker and selective genes, but instead to apply molecular diagnostic techniques and in the case of success to clone transgenic plants obtained.

Despite these concerns, in 2006 the world's first plant-made vaccine candidate for Newcastle disease in chickens, produced in a suspension-cultured tobacco cell line by Dow Agro Science (IN, USA), was registered and approved by the US Department of Agriculture (USDA). In addition, two plant-made pharmaceuticals are moving through Phase II and Phase III human clinical trials. Biolex's (NC, USA) product candidate, Locteron®, is in Phase IIb clinical testing for the treatment of chronic hepatitis CA [101] and a Phase III clinical trial was recently completed with Protalix's (Carmiel, Israel) product UPLYSO, a plant cell expressed recombinant glucocerebrosidase enzyme to cure Gaucher's disease [17,102].

Recently, two new-generation vaccines were developed against HIV-1 and were used in clinical trials. One clinical trial (Vaxgen [CA, USA]) was performed with a vaccine based on the p120 subunit (HIV-1); the vaccine was able to induce a real immune response and binding of virions with neutralizing antibodies but did not induced a broad immune reactivity. The second trial was

performed with a vaccine based on a recombinant adenovirus vector (rAd5) expressing Gag, Pol and Nef of HIV-1 (Merck [NJ, USA]). During the trial conducted by Merck and the US NIH, the recombinant adenovirus interacted with pre-existing adenoviruses and helped the entrance of HIV virions in tested African volunteers tested [80]. This raised numerous debates and doubts regarding the possibility of creating new-generation vaccines against HIV-1. It was said that the failures in vaccine design were provoked by insufficient knowledge of the nature of HIV-1 and of the interaction of the virus with immune cells.

Later, a team of Russian scientists designed a new generation HIV vaccine based on 86 different epitopes of HIV to induce the synthesis of broad-spectrum neutralizing antibodies against HIV [81]. These sequences were carefully selected after comparison with well-known sequences in the human genome. Many epitopes were considered for the creation of synthetic (chimeric) sequences of the TCI (T- and C-cell immunogens) protein that were able to neutralize mutated and escaped virions. Up untill now, studies investigating the use of this chimeric gene for the creation of mucosal vaccine have not been performed.

Recently, several investigations are focusing on the interaction of HIV with other associated viruses. Hepatitis B and C viruses, papillomavirus and Kaposi's sarcoma virus have many similar features [82]; one of them is the ability to spread via sexual contact and to invade the host via the mucosal surface route. Therefore, it could be possible to create multicomponent vaccines against three viruses by using immunogenic epitopes of HIV-1 (gp120 or chimeric polyepitopic gene T-cell immunogen), antigenic protein PreS2-S HBV and HPV L1. It may be assumed that using three immunogenic proteins could induce broad-spectrum neutralizing antibodies. For example, using the Forest Semliki virus for the expression of gp140 HIV-1 resulted in significant increases in the synthesis of neutralizing antibodies against HIV-1 [83].

The hope is that all the successful results obtained in the last few years in these fields will also increase the interest towards the production of mucosal vaccines in transgenic plants.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Key issues

- Mucosal vaccination is a perspective for the control of infectious diseases, since it is capable of inducing humoral and cell-mediated responses.
- Transgenic plants can be used as bioreactors for the production of subunit, mucosally delivered protective antigens, as the plant cell walls protect the antigen from the acidic environment of the stomach and enable intact antigen to reach the gut-associated lymphoid tissue. In the case of encapsulated vaccines, capsules should be prepared from material that protects the antigen from the adverse conditions of the GI tract.
- Several expression systems can be used for the production of antigens in plants.
- Transgenic plants are very useful to produce mucosal vaccines against widespread infectious diseases such as TB, hepatitis B and HIV.
- Technical problems hindering the development of this technology, such as increasing the immunogenicity of plant-made delivered vaccines and downstream processing, are now being resolved.

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