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REVIEW

Two decades of plant-based candidate vaccines: a review of the chimeric protein approaches

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Abstract Genetic engineering revolutionized the concept of traditional vaccines since subunit vaccines became reality. Additionally, over the past two decades plant-derived antigens have been studied as potential vaccines with several advantages, including low cost and convenient administration. More specifically, genetic fusions allowed the expression of fusion proteins carrying two or more components with the aim to elicit immune responses against different targets, including antigens from distinct pathogens or strains. This review aims to provide an update in the field of the production of plant-based vaccine, focusing on those approaches based on the production of chimeric proteins comprising antigens from human pathogens, emphasizing the case of cholera toxin/*E. coli* enterotoxin fusions, chimeric viruses like particles approaches as well as the possible use of adjuvant-producing plants as expression hosts. Challenges for the near future in this field are also discussed.

Keywords Plant-based vaccine · Fusion protein · Broad immune protection · Molecular farming · Oral immunization

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Introduction

Jenner and Pasteur, the pioneers of vaccination in the 18th and 19th centuries, used attenuated or inactivated microorganisms to induce protective immunity to several pathogens. Their techniques, although crude, were successful to control many infectious diseases. However, in the recent decades, the emergence of new pathogens, such as HIV, has highlighted the need for continued development and improvement of vaccination strategies. Despite the efficiency of those early vaccines, there is concern over their safety, because they often have residual toxicity following inactivation and might contain toxic components, such as lipopolysaccharides (Ryan et al. 2001; Lavelle 2005).

This has changed the focus of vaccine development to safer although often less immunogenic, subunit vaccines composed of purified antigenic components of the microorganism. The biotechnological revolution made the large-scale production of recombinant proteins possible enabling the manufacture of these subunit vaccines. Molecular cloning techniques as well as improvements on gene synthesis have opened new doors in the rational design of subunit vaccines (Ryan et al. 2001; Lavelle 2005). In particular, chimeric proteins carrying epitopes from different pathogens, linkers, or adjuvant sequences offers not only increased immunogenicity of the recombinant antigen but also the possibility to elicit a broad cellular or humoral immune response (Berzofsky et al. 2001).

This can be obtained by selecting the epitopes capable of eliciting neutralizing antibodies. In addition, the epitopes must be conserved and preferably have to be linear and immunogenic per se. This approach also opened opportunities for the design of polyvalent vaccines trough the design and production of chimeras-carrying epitopes

from different pathogens, strains or even different isolates. A number of models have demonstrated the feasibility to elicit broad immune responses using such a type of molecules (Berzofsky et al. 2001; Karlsson Hedestam et al. 2008). However, the formulations based on purified or recombinant subunit vaccines, are often poorly immunogenic and require adjuvants to help stimulate protective immunity.

Transgenic technologies have led to relevant application mainly in agriculture. Throughout the decade of the 90s, the concept of plant-based vaccines emerged as one of these applications. Plants would be used as production platform of well-characterized immunoprotective antigens and also as delivered vehicles. Plant-based vaccines attracted the attention of many research groups as this approach offers the advantages of mucosal immunization, along with other features, such as no requirement for fermentation, stability, low cost and a few side-effects; additionally, when the antigenic protein is produced in the edible parts of the plant, such as grain, fruit or even leaves, they may not require purification (Howard 2005). The first attempt to explore this biotechnological approach were directed to prove the principle that plants are capable to express functional full length antigens (Paul and Ma 2010). Veterinary field has also been exploring plant-based vaccine models against several pathogens, including the porcine transmissible gastroenteritis virus (Hammond and Nemchinov 2009), the highly pathogenic avian influenza virus (Kalthoff et al. 2010), additionally the case of an antibody fragment for passive immunity against gastrointestinal parasitic infections in chickens was reported (Zimmermann et al. 2009).

The production of chimeric proteins became the next logical focus for the development of plant-derived vaccines, with the advantages inherent to this approach. To date several reports have assessed the plant production of chimeric proteins as potential vaccines. This review describes the current status in the field, particularly those efforts to assess the expression of fusion proteins in order to produce vaccine candidates with broader immune protection in humans.

Multiepitopic protein design

The optimal vaccine against infections should be designed to selectively induce mainly protective immune responses and avoid undesirable ones. Such a vaccine must not therefore mimic the natural infection, but rather be engineered to contain only selected epitopes. The epitope selection must be based on their ability to induce protective immunity. Thus, in the rational design of artificial vaccines it is be necessary to identify appropriate epitopes that elicit protective helper and cytotoxic T cell and neutralizing antibody responses as well as to avoid epitopes that elicit harmful responses (Berzofsky et al. 2001; Karlsson Hedestam et al. 2008). Configuration of epitopes in a single polypeptide or protein is a critical factor for the immunogenic properties. This feature is almost only determined empirically although some softwares are available to identify potential epitopes as well as the protein structure of the desired chimeric protein.

The selection of linkers is particularly relevant in the design of functional chimeric proteins since they can play an important role on displaying specific epitopes in the overall structure of the fusion protein. Besides appropriate amino acid composition, the overall folding of the linker must be considered. According to Robinson and Sauer (1998) linkers have a significant effect on protein folding stability. It is undesirable to have a linker sequence with a high propensity to form α-helical or β-strand structures since they would limit the flexibility of the fusion protein and consequently affect its functional activity. The design of a linker sequence often requires careful consideration in order to avoid such secondary structural elements. Proline-containing sequences are, for example, often used as linkers to disrupt secondary structures allowing the display of epitopes fused at the end of the protein (Cardenas and Clements 1993). Many studies of linker peptides in various protein families have come to the conclusion that linkers lack regular secondary structure: they display varying degrees of flexibility to match their particular biological purpose and are rich in Ala, Pro and charged residues. Unfortunately, there are no reliable selection criteria available for using linker design. Most current linker design selection processes are still largely dependent on intuition. Although significant progress has been made in predicting secondary structures of proteins based on primary sequences, our understanding of sequence structure correlation is still limited (Crasto and Feng 2000).

Current status of chimeric protein approaches

Fusion proteins based on CT/LT

Cholera toxin (CT) and heat labile enterotoxin from ETEC (LT) are highly similar in both structure and function since both are ADP-ribosylating exotoxins (Spangler 1992). They consist of a receptor binding (B) oligomer formed by five identical B subunits (CTB/LTB) and an enzymatic subunit (CTA/LTA). CTB and LTB are highly immunogenic by oral route and lack toxicity (Field 1979). These features make CTB and LTB protein carriers attractive for the oral delivery of genetically attached antigens and epitopes (reviewed by Nashar et al. 1993). Using conventional microbial systems these proteins have been extensively

used as carriers for unrelated antigens resulting in the induction of immune responses by mucosal routes (Reviewed by Nashar et al. 1993). This section summarized plant-based vaccination approaches based on the use of these carriers (Table 1).

Enteric diseases

Diarrhea caused by *Vibrio cholera*, enterotoxigenic *Escherichia coli* (ETEC) and rotavirus constitutes a major health issue worldwide, responsible for the highest levels of child morbidity and mortality in the developing countries (Schaetti 2009).

Yu and Langridge (2001) described an early attempt to express two chimeric proteins in potato, one comprising of CTB and the 22-amino acid immunodominant epitope of the murine rotavirus enterotoxin NSP4 while the second one consisted of the CTA2 and the ETEC fimbrial colonization factor CFA/I. The assembling of cholera holotoxin-like structures that retained the ability to bind the GM1 receptor was observed. However, cholera toxin neutralization assays were not performed, only protection was predicted based on the antibody titers. Later, this research group reported additional functional studies where the chimeric protein was able to induce antibodies capable to protect $Caco_2$ cells against the binding of toxic *E. coli*, providing more evidence about the feasibility of plant-based multi-component vaccine protection against enterotoxigenic *E. coli* and rotavirus (Lee et al. 2004).

LTB has also been used as carrier for the heat stable toxin (ST) from ETEC. In a genetic fusion, ST was fused to the C-terminus of LTB (LTB::ST) and expressed in tobacco chloroplasts. Pentamers were detected by GM1-ELISA assay and anti-LTB humoral responses were triggered by feeding mice with tobacco leaves. Moreover, challenge with CT showed that mice immunized by the oral route were protected against CT activity (Rosales-Mendoza et al. 2009). A nuclear-encoded LTB:ST protein was also produced in tobacco, showing to be immunogenic at low doses which could be attributed to glycosilation and should be further analyzed (Rosales-Mendoza et al. 2011). Epitopes P4 and P6 from toxin co-regulated pilus subunit A (TCPA) from *Vibrio cholerae* were fused to CTB and expressed in tomato plants by Sharma et al. (2008b). Western blot and GM1-ELISA probed the synthesis and assembly of pentameric chimeric proteins in tomato fruit tissue. Immunization data was not yet presented, however. This approach has implications on the development of an efficient vaccine capable of inducing anti-toxin as well as anti-colonizing immunity against *Vibrio cholerae* infections.

Sharma et al. (2008a) reported the expression of the accessory colonization factor (*acf*) subunit A fused to CTB in tomato. Expression of the genetic fusion was confirmed, but only dimeric and trimeric forms were detected by Western blots under non-reducing conditions. The binding of CTB::VP7 fusion protein pentamers to GM1 ganglioside receptors was detected by enzyme-linked immunosorbent assay (ELISA). The feasibility of using these plants for the delivery of this rotavirus capsid protein antigen remains to be tested in animal models. Choi et al. (2005) obtained a genetic fusion comprising the coding regions for VP7 from rotavirus, the outer capsid protein of simian rotavirus SA11 and CTB. Potato transgenic plants were able to produce a pentameric CTB::VP7 fusion protein confirmed by GM1-ELISA. Although no immunization data was presented, these results demonstrated the ability of plant cells to produce the CTB-based chimeric proteins for rotavirus capsid antigens.

Human immunodeficiency virus

Infection with HIV, the causative agent of AIDS, is responsible for a large number of deaths every year worldwide and represents a significant threat to human health. Multiple HIV genes and proteins have been evaluated as candidate for HIV vaccines. Targets for vaccines include structural proteins (gag, env, gp120, gp41, and gp160), viral enzymes (pol), and regulatory proteins (nef, tat, rev and vpr). Matoba et al. (2009) explored the expression in *Nicotiana benthamiana* plants of chimeric protein CTB with aa 649-684 from the membrane proximal region (MPR) of the gp41 envelope protein, which is relevant since it plays a central role in several of the suggested mucosal HIV transmission pathways. The report provided evidence that GM1-binding pentameric CTB–$MPR_{649–684}$ were assembled and triggered anti-$MPR_{649–684}$ antibodies in a mouse model under a mucosal prime-systemic boost regimen.

The simian-human immunodeficiency virus (SHIV 89.6p) Tat regulatory element protein was fused to the c-terminus of the cholera toxin B subunit gene (ctxB-tat) and introduced into *Solanum tuberosum* cells. Oligomeric structures of pentamer size were detected in transformed tuber extracts by immunoblot analysis. The binding of CTB-Tat fusion protein pentamers to GM1 was proved by GM1-ELISA. The biological activity of this protein would indicate the feasibility of using this fusion against HIV-1 infection (Kim et al. 2004d).

CTB was fused to the simian immunodeficiency virus (SIVmac) Gag p27 capsid gene. The fusion CTB-Gag was produced in potato plants. Synthesis and assembly of the CTB-Gag fusion protein into oligomeric structures of pentamer size was confirmed by GM1-ELISA. Functionality of the carrier and adjuvant properties of CTB in a plant-based formulation subunit against SIV in macaques remains to be tested (Kim et al. 2004c).

Table 1 CTB and LTB fusion proteins reported as candidate plant-based vaccines for human

Chimeric protein	Vaccine antigen	Linker sequence	Plant host	Expression system	Expression levels	Functionality assays	References
CTB::AMA1	Malarial apical membrane antigen-1	GPGP	Tobacco and Lettuce	Chloroplast-derived	13.17% TSP in tobacco and 7.3% TSP in lettuce	Systemic and mucosal immune response in mice. Oral and subcutaneous immunization protected mice from CT challenge	Davoodi-Semiromi et al. (2010)
CTB::MSP1	Malarial merozoite surface protein-1	GPGP	Tobacco and Lettuce	Chloroplast-derived	10.11% TSP in tobacco and 6.1% TSP in lettuce	Systemic and mucosal immune response in mice. Oral and subcutaneous immunization protected mice from CT challenge	Davoodi-Semiromi et al. (2010)
CTB::$MPR_{649–684}$	Membrane proximal region of gp41 of HIV	GPGP	*Nicotiana benthamiana*	Nuclear-derived	0.01–0.2% TSP	Systemic and mucosal immune response in mice	Matoba et al. (2009)
CTB::As14	Antigen protective of *Ascaris suum*	GPGP	Rice	Nuclear-derived	1.5 μg/g seed	Systemic immune response in mice	Matsumoto et al. (2009)
CTB::As16	Antigen protective of *Ascaris suum*	GPGP	Rice	Nuclear-derived	50 μg/g seed	Not reported	Nozoye et al. (2009)
CTB::P4 CTB::P6	Epitopes of toxin co-regulated pilus subunit A of *Vibrio Cholerae*	GPGP	Tomato	Nuclear-derived	0.17%–0.096% TSP	Not reported	Sharma et al. (2008b)
CTB::ACFA	Accessory colonization factor subunit A of *Vibrio Cholerae*	GPGP	Tomato	Nuclear-derived	0.006-0.25% TSP	Not reported	Sharma et al. (2008a)
CTB-Pins	Subunit–human proinsulin	GPGP	Tobacco and Lettuce	Chloroplast-derived	16% TSP in tobacco and 2.5% TSP in lettuce	Systemic immune response in mice	Ruhlman et al. (2007)
CTB::$FimA_{266-337}$	*Porphyromonas gingivalis* antigen	GPGP	Potato	Nuclear-derived	0.13 -0.33% TSP	Not reported	Shin et al. (2006)
CTB::InsB3	B chain of human insulin	VE	Tobacco	Nuclear-derived	0.11% TSP	Not reported	Li et al. (2006)
CTB::VP7	Glycoprotein VP7 of simian rotavirus	GPGP	Potato	Nuclear-derived	0.01% TSP	Not reported	Choi et al. (2005)
CTB::Gag	Gag p27 capsid gene of Simian Immunodeficiency Virus	GPGP	Potato	Nuclear-derived	0.016–0.022% TSP	Not reported	Kim et al. (2004c)
CTB–NSP4::CFA/I–CTA2	Enterotoxigenic *E. coli* fimbrial antigen and rotavirus epitope	GPGP	Potato	Nuclear-derived	0.002% TSP	Systemic immune response in mice	Lee et al. (2004)
CTB::LF	Domain I of anthrax lethal factor protein	GPGP	Potato	Nuclear-derived	0.002% TSP	Not reported	Kim et al. (2004a)
CTB::gp120	Envelope glycoprotein gp120 of HIV	GPGP	Potato	Nuclear-derived	0.002–0.004% TSP	Not reported	Kim et al. (2004b)
CTB::Tat	Tat regulatory element protein of VIH	GPGP	Potato	Nuclear-derived	0.005–0.007% TSP	Not reported	Kim et al. (2004d)
CTB::NSP4, CTB::CFA I	Enterotoxigenic *E. coli* fimbrial antigen and rotavirus epitope	GPGP	Potato	Nuclear-derived	0.01–0.1% TSP	Systemic and mucosal immune response in mice. Reduced symptoms following rotavirus challenge in neonates mouse	Yu and Langridge (2001)

Table 1 continued

Chimeric protein	Vaccine antigen	Linker sequence	Plant host	Expression system	Expression levels	Functionality assays	References
LTB::ST	Heat-stable toxin of ETEC	HDPRVPSS	Tobacco	Chloroplast-derived	2.3% TSP	Systemic and mucosal immune response in mice. Oral immunization protected mice from CT challenge	Rosales-Mendoza et al. (2009)
LTB::ST	Heat-stable toxin of ETEC	HDPRVPSS	Tobacco	Nuclear-derived	0.05% TSP	Systemic and mucosal immune response in mice	Rosales-Mendoza et al. (2011)
LTB::ESAT-6	Tuberculosis antigen	SNPHVPN	*Arabidopsis thaliana*	Nuclear-derived	11–24.5 μg/g FW	Not reported	Rigano et al. (2004)
LTB::ZP3	Immunocontraceptive epitope	NSDPHVPN	Tomato	Nuclear-derived	37.8 mg/g DW	Not reported	Walmsley et al. (2003)

The V3 loop has been produced as a fusion protein based on CTB using potato plants as host and the CTB-gp120 fusion protein was detected as oligomeric structures of pentamer size. GM1-ELISA analysis revealed expression levels ranging 0.002–0.004% TSP as well as GM1 binding ability (Kim et al. 2004b). The outer capsid protein of simian rotavirus SA11 had also been expressed through fusion with the carboxyl terminus of CTB. The transgenic *Solanum tuberosum* plants showed ability to synthesize and assemble the CTB::VP7 fusion protein into oligomers of pentameric size.

Tuberculosis

Components from *M. tuberculosis,* as the early secretory antigenic target, ESAT-6, and the antigen 85B (Ag85B) are antigens with vaccine potential. Rigano et al. (2004) reported the expression of *Arabidopsis*-derived tuberculosis (TB) antigen ESAT-6 fused to LTB. Plant material was fed to mice, inducing antigen-specific Th1 responses reporting for the first time an orally delivered, subunit, tuberculosis vaccine priming an antigen-specific response (Rigano et al. 2006).

Floss et al. (2010) have reported the production of a Ag85B:ESAT-6 fusion protein in tobacco plants by means of the elastin-like peptide (ELP) fusion strategy, which targets the protein with VPGVP motif derived from native mammalian elastins, facilitating purification by a procedure called inverse transition cycling. The level of production for this TBAg-ELP fusion protein reached up to 4% of the tobacco leaf total soluble proteins and it was able to trigger host antibodies and T cells recognizing mycobacterial Ag85B and probably ESAT-6 in mice and piglets immunized with purified TBAg-ELP or with crude tobacco leaf extracts.

Parasitic infections

The protective antigen As16 from the roundworm *Ascaris suum* has also been expressed as CTB fusion protein. This pathogen is a gastrointestinal nematode infecting both humans and animals, and the infection is endemic in many parts of the world (Crompton 2001). The plant model was rice and the transgenic seeds elicited in mice-specific serum antibody response when CT was used as adjuvant. Moreover, challenge experiment proved that immunized mice had a lower lung worm burden than control mice, suggesting that the rice-derived chimeric protein is a promising approach as an edible vaccine for controlling parasitic infections. Similar report was published for a CTB As14, however, no immunization experiment was presented (Nozoye et al. 2009).

Immunocontraceptive approach

Transgenic tomato plants were developed by Walmsley et al. (2003) to express a fusion protein consisting of the LTB and the ZP3 immunocontraceptive epitope. The fusion protein assembled pentamers, however, immunogenicity of the chimeric protein has not been reported so far.

Other target diseases

The 27 kDa domain I of anthrax lethal factor protein (LF), was expressed by Kim et al. (2004a) as fusion protein consisting of CTB to which carboxyl terminus fused to the antigen of interest. Potato plants (*Solanum tuberosum*) were transformed and immunoblot revealed the presence of the corresponding recombinant protein showing reactivity to both anti-CTB and anti-LF primary antibodies, thus verifying the synthesis and assembly of biologically active CTB-LF fusion protein oligomers. Using these plants to deliver adjuvant LF protein for CTB-mediated immunostimulation of mucosal immune responses against anthrax toxin remains to be tested (Kim et al. 2004a).

Fusion proteins based on VLPs

Another potential strategy to produce HIV vaccines is the use of VLP (virus-like particles) as fusion platforms for the mucosal delivery of heterologous antigens. The VLP have the ability to stimulate strong immune responses upon oral delivery, moreover, it has been proposed that the compact and highly ordered structures of VLP can provide resistance to digestive proteases (Huang et al. 2005). Among the main sequences used to produce VLP are the antigenic proteins for the hepatitis B virus (HBV). It was widely demonstrated that HBV VLP carrier spontaneously assembles in plant cells, giving VLPs with a preserved structure (Smith et al. 2003). See Table 2.

In 2007, a peptide containing eight epitopes of the proteins Gag and Pol from the HIV-1 (Michel et al. 2007) were produced through fusion with the surface protein of HBV in order to produce HIV-1::HBV VLPs. The recombinant protein was successfully expressed in *Nicotiana tabacum* and *Arabidopisis thaliana*. In both expression systems, HBV VLPs carrying the HIV-1 polypeptide on their surface were obtained, with comparable production levels (Greco et al. 2007).

Shchelkunov et al. (2006) described the development of tomato transgenic plants that produce a chimeric HBS::TBI containing the surface protein of hepatitis B virus (HBS) and Env and Gag epitopes of human immunodeficiency virus (TBI). Their studies demonstrate that oral administration of dried tomato tissue containing the chimeric

Table 2 VLP fusion proteins reported as candidate plant-based vaccines for human

Chimeric protein	Vaccine antigen	Linker sequence	Plant host	Expression system	Expression levels	Functionality assay	References
HBcore:: DV2d3	Dengue virus serotype 2 domain III	GTGGGGS	*Nicotiana benthamiana*	Magnifection	2.4 mg/g FW	Not reported	Martínez et al. (2010)
HBsA :: preS1	Hepatocyte receptor-binding presurface 1 region	Not reported	Rice	Nuclear-derived	31.5 ng/g DW	Systemic immune response in mice	Qian et al. (2008)
HB::PA-D4	Domain-4 epitope of the anthrax protective antigen	Not reported	Tobacco	Nuclear-derived	Not reported	Systemic immune response in mice. Assembling of virus-like particles (VLP) was not observed	Bandurska et al. (2008)
HBV::HIV-1	HIV-1 antigen	Not reported	Tobacco and *Arabidopsis thaliana*	Nuclear-derived	2–26 ng/g tobacco FW 3–15 ng/g *Arabidopsis* FW	Not reported	Greco et al. (2007)
HBS::TBI	Env and Gag epitopes of Human Immunodeficiency Virus (HIV-1)	Not reported	Tomato	Nuclear-derived	0.3 ng/mg DW	Systemic and mucosal immune response in mice	Shchelkunov et al. (2006)

HBS::TBI can elicit both serum and mucosal immune responses in mice. All of these results support the production of an efficient HIV::HBV bivalent vaccine against hepatitis B and the acquired immunodeficiency syndrome (AIDS), which could be particularly useful in the developing countries where the two diseases pose major public health issues.

Recently Martínez et al. (2010) produced in *Nicotiana benthamiana* plants, a fusion HB core protein, carrying a truncated version of the E-protein from Dengue virus. The antigenicity of the recombinant protein was proved trough the recognition by anti-E antibody, but its immunogenicity has not yet been assessed.

F1–V fusion proteins

Yersinia pestis, a Gram negative bacterium, is a dangerous pathogen that causes severe infections in humans: when inhaled, it can quickly induce fatal pneumonic plague; it still represents a serious public health threat in various parts of the world as well as a potential agent in bioterrorism (Prentice and Rahalison 2007; Titball and Leary 1998). Even though living and inactivated whole-cell vaccines are available for humans, serious drawbacks limit their use for prevention of outbreaks (Williamson et al. 1997). Thus, there is a need for stable, safe, and easily administered mucosal vaccines capable of eliciting effective protection against *Y. pestis* infections.

Two *Y. pestis* proteins, F1 and V, are known to be effective immunogens and have been proposed as candidates for a combined subunit vaccine against plague (Williamson et al. 1995). The fraction 1 capsular protein (F1) is a glycoprotein which is the major component of the *Y. pestis* polysaccharide capsule and is one of the primary antigens used in vaccines against plague because it has showed extremely high immunogenicity in both animals and humans. F1 is considered an important but not essential virulent factor unique to this pathogen (Andrews et al. 1996; Williamson et al. 1995; Welkos et al. 1995). The V antigen (LcrV) includes one subunit of the *Y. pestis* type III secretion system and is a second antigen commonly used for *Y. pestis* vaccines. It has been reported that V antigen has immunosuppressive potential by stimulating IL-10 secretion (Sing et al. 2002; Anderson et al. 1996).

A number of experimental vaccines produced in plants have been developed for plague infection (see Table 3). In the first approach, F1::V fusion antigens were expressed in tomato fruit. The immunogenicity of the recombinant protein was confirmed in mice that were subcutaneously primed with bacterially produced F1::V and then boosted orally with transgenic tomato fruit. It is noteworthy that, although nuclear transformation was employed, high concentrations of the fusion protein F1–V was achieved

Table 3 F1::V fusion proteins reported as candidate plant-based vaccines for human

Chimeric protein	Vaccine antigen	Linker sequence	Plant host	Expression system	Expression levels	Functionality assays	References
F1::V	*Y. pestis antigens*	Not reported	Lettuce	Nuclear-derived	0.08% TSP	Systemic immune response in mice	Rosales-Mendoza et al. (2010a)
F1::V	*Y. pestis antigens*	Not reported	Carrot	Nuclear-derived	0.3% TSP	Systemic immune response in mice	Rosales-Mendoza et al. (2010b)
F1::V	*Y. pestis antigens*	Not reported	*Nicotiana tabacum* NT1 cells	Nuclear-derived	0.4–0.9% TSP	Not reported	Alvarez and Cardineau (2010)
F1::V	*Y. pestis antigens*	Not reported	*Medicago sativa*	Nuclear-derived	0.015–0.023% TSP	Not reported	Alvarez and Cardineau (2010)
F1::V::LicKM	*Y. pestis antigens*	Not reported	*Nicotiana benthamiana*	Agro-infiltration	Not reported	Systemic immune response and protection against lethal challenge with *Y. pestis in Cynomolgus macaques*	Chichester et al. (2009)
F1::V	*Y. pestis antigens*	Not reported	Tobacco	Chloroplast-derived	14.8% TSP	Systemic immune response in mice and protection against challenge with *Y. pestis* in mice	Arlen et al. (2008)
F1:: LicKM LcrV:: LicKM	*Y. pestis* antigens	Not reported	*Nicotiana benthamiana*	Agro-infiltration	Not reported	Systemic immune response and protection against lethal challenge with *Y. pestis in Cynomolgus macaques*	Mett et al. (2007)
F1::V	*Y. pestis antigens*	Not reported	Tomato	Nuclear-derived	4–10% TSP	Systemic immune response in mice	Alvarez et al. (2006)

(4–10% of TSP) in green freeze-dried tomato fruit (Alvarez et al. 2006).

The same fusion protein has also been expressed in *Nicotiana tabacum* NT1 cells, *Medicago sativa*, carrot and lettuce, however, immune response in mice was only reported for the F1::V produced in carrot and lettuce (Alvarez and Cardineau 2010; Rosales-Mendoza et al. 2010a; Rosales-Mendoza et al. 2010b). In 2007, Mett et al. (2007) produced in *Nicotiana benthamiana*, F1 and V that were independently fused to a carrier molecule, LicKM, which is a thermostable enzyme lichenase from *Clostridium thermocellum*. The produced candidate vaccine conferred protection to monkeys against lethal challenge with *Y. pestis.*

A transplastomic approach was also pursued by Arlen et al. (2008) that revealed F1::V was expressed in tobacco plants; high levels of accumulation were reached (14% TSP) and it was immunogenic when administered orally to mice. Moreover, this plant-derived oral vaccine protected mice from live *Y. pestis* challenge. Another interesting approach was reported by Chichester et al. (2009). The F1::V fusion linked to the carrier molecule LicKM expressed in *Nicotiana benthamiana* was able to elicit systemic immune response protecting against *Y. pestis* challenge in macaques (*Cynomolgus macaques*). All of these results support the efficiency of plants as a production platform for plague vaccines.

HIV chimeric proteins

HIV antigens have been targeted in the form of a number of chimeric proteins (Table 4). The use of Ig fusion partners was proposed as a strategy for increasing the yield of a recombinant protein in plants (Obregon et al. 2006). HIV p24 antigen was expressed as a genetic fusion with the α-2 and α-3 constant region sequences from human Ig-chain in tobacco leaves. Immunological studies showed that the p24::IgA fusion protein was immunogenic in mice, suggesting a first step in the development of a new strategy for HIV vaccine at low cost and high yield. On the other hand, using a transplastomic approach, Zhou et al. (2008) reported the expression of two candidate HIV-1 antigens, p24 and Nef. This fusion protein was successfully expressed at high levels in plastids of tobacco and tomato. However, it has yet to be demonstrated that the chloroplast-derived HIV-1 antigens are able to induce neutralizing antibodies against HIV strains.

Chimeras targeting tetanus, diphtheria and pertussis

Vaccination against tetanus, diphtheria and pertussis (DPT) has markedly reduced the number of cases and deaths produced by the exotoxins of *Corynebacterium diphteriae*, *Bordetella pertussis* and *Clostridium tetani*. The first approach against a DPT vaccine became available during the 1920s and was composed of diphtheria and tetanus toxoids and the whole cell pertussis, however, due to this composition, several complications of the disease and secondary effects were frequent. In 1991, less reactogenic acellular pertussis vaccine (DTaP) was licensed (CDC 1992). Prompted by the local adverse reactions and reduced acceptance of the DTaP vaccine in adults, a simplified vaccine against these bacterial childhood diseases was formulated. It consists of a chimeric protein named sDPT made of two immunodominant protective epitopes from each one of the following: tetanus toxin heavy chain from *C. tetani*, the pertussis toxin subunit 1 of *B., pertussis* and diphtheria toxin subunit A of *C. diphteriae* (Soria-Guerra et al. 2007). This fusion protein was expressed in tomato plants by *Agrobacterium* mediated transformation as a first step to assess the functionality. Two adjuvant coding sequences from tetanus toxin and the endoplasmic retention signal SEKDEL were also included. Although the expression of the polypeptide was active in vitro, the expression levels in tomato plants reached 0.01% TSP (Soria-Guerra et al. 2007). Despite this relative low protein accumulation, a specific antibody response against the targetted antigens were detected in serum, gut, and lung samples from mice dosed orally with transgenic tomatoes (Soria-Guerra et al. 2011).

In order to enhance these expression levels, a transplastomic approach was conducted. A synthetic gene encoding for the DPT polypeptide was re-designed in order to optimize its expression in tobacco chloroplasts. Using biolistic-mediated transformation, several transformants were confirmed in the T1 progeny. The DPT protein content in tobacco leaves was 0.8% of TSP, which was approximately 110 times higher than that detected in tomato plants. Furthermore, it was showed that this candidate plant-based vaccine was immunogenic in mice by the oral route (Soria-Guerra et al. 2009). Although these results demonstrate the feasibility of plants as a platform for the generation of a recombinant subunit DTP vaccine, it is still necessary to perform toxin neutralization assays as well as challenges with each of the exotoxin in order to prove if protective immunity can be achieved.

Other potential dual vaccines

Studies on a prospective candidate for anthrax vaccination have also been conducted. In 2010, Andrianov et al. reported the production of chimeric recombinant protein (immunoadhesin) made of functional anthrax toxin receptor (ATR) domain fused with the human immunoglobulin Fc fragment (pATR-Fc). Previous works showed that the fusion of heterologous peptides with Fc provides better

Table 4 Other potential vaccines reported as candidate plant-based vaccines for human

Chimeric protein	Vaccine antigen	Linker sequence	Plant host	Expression system	Expression levels	Functionality assays	References
p24::Nef Nef::p24	HIV-1 antigens	Not reported	Tobacco and tomato	Chloroplast-derived	40% TSP	Not reported	Zhou et al. (2008)
p24 :: IgA	HIV-1 antigens	Not reported	Tobacco	Nuclear-derived	0.88–1.4% TSP	Systemic immune response in mice	Obregon et al. (2006)
E7::LicKM	Human Papilloma Virus antigen	Not reported	*Nicotiana benthamiana*	Nuclear-derived	400 mg/kg FW	Systemic immune response and protection against challenge with E7-expressing tumor cells in mice	Massa et al. (2007)
DPT	Epitopes of *Corynebacterium diphtheriae*, *Bordetella pertssis* and *Clostridium tetani*	EQIAD SVQ GDNA KEH VSEEKAKQYL	Tobacco	Chloroplast-derived	0.8% TSP	Systemic and mucosal immune response in mice	Soria-Guerra et al. (2009)
DPT	Epitopes of *Corynebacterium diphtheriae*, *Bordetella pertssis* and *Clostridium tetani*	EQIAD SVQ GDNA KEH VSEEKAKQYL	Tomato	Nuclear-derived	4.5–17 μg/g FW	Systemic and mucosal immune response in mice	Soria-Guerra et al. (2007, 2011)
pATR::Fc	Anthrax toxin receptor fused with human antibody Fc region	None	Tobacco	Nuclear-derived	3 mg/kg FW	Neutralization of anthrax toxin in vitro	Andrianov et al. (2010)
TBAg::ELP	Ag85B and ESAT-6 antigens of *Mycobacterium tuberculosis*	Not reported	Tobacco	Agro infiltration	4% TSP	Systemic immune response in mice and piglets	Floss et al. (2010)

yields, stability, and facilitates purification process in plants (Obregon et al. 2006; Ashkenazi and Chamow 1997). It was observed that the recombinant protein protected J774A1 macrophage cells against the anthrax toxin, which suggests that plant-derived pATR-Fc antibody-like protein is suitable for post-infection therapy of anthrax. Likewise, a fusion protein comprising the epitope from domain-4 of the anthrax protective antigen (PA-D4) and the hepatitis B core antigen (HBcAg) was expressed in *Nicotiana tabacum* leaves. The recombinant protein was able to induce a systemic immune response; nevertheless, assembling of virus-like particles (VLP) was not observed (Bandurska et al. 2008). Among carrier proteins used in this field, elastin-like peptides (ELP) and a thermostable enzyme lichenase from *Clostridium thermocellum* (LicKM) yield promising results. Previous works have showed that ELP-fusions enhance the expression of many recombinant proteins and also have the advantage of an easy and inexpensive purification procedure due to the property of a thermally responsive reversible phase transition which avoids the cost-intensive affinity chromatography (Floss et al. 2009; Patel et al. 2007; Scheller et al. 2004).

The ELP plant-based expression strategy was successfully employed by Floss et al. (2010) in an approach to develop a subunit vaccine candidate against tuberculosis (TB). They produced the mycobacterial antigens Ag85B/ESAT- 6 (TBAg) as an ELP fusion in tobacco plants. The immunogenicity and safety of the plant expressed TBAg were confirmed in both mouse and piglet models. A vaccine against this disease would have an enormous impact on developing countries. In the same way, the E7 oncoprotein from Human Papilloma Virus (HPV) was fused to LicKM (Massa et al. 2007) with the aim of developing a candidate for cancer vaccine. The recombinant protein was expressed in *N. benthamiana* leaves and the immunogenicity and prophylactic potential of plant-produced LicKM::E7 was tested in mice. The reported results support the concept of producing an anti-tumor vaccine in plants.

In order to study the potential of plant expression systems to produce a candidate for subunit vaccine against dengue virus, the critical domain III serotype 2 of Dengue virus (DV2d3) was genetically fused to hepatitis B core antigen (HBcore) and expressed in *N. benthamiana* plants using the Magnifection system. This approach allows very fast production and high recombinant protein expression levels (Gleba et al. 2005a). The fusion DV2d3::HB core was produced at levels around 7% of TSP; furthermore, the protein was able to self-assembly into virus like-particles (VLPs), however, the immunological properties of the recombinant protein have yet to be analyzed (Martínez et al. 2010).

Another approach to produce a novel hepatitis B vaccine in plants was reported by Qian et al. (2008); they demonstrated that rice seeds are a suitable system for the production of the recombinant protein, containing the hepatocyte receptor-binding presurface 1 region (preS1) fused to major HBV surface protein (HBsA). They also demonstrated that the recombinant HBsA::preS1 produced in the transgenic seeds can assemble into VLP structures and induce immunological response in mice, thus implying that this rice-derived protein might have potential as a novel, oral, HBV candidate vaccine.

Immunological considerations

The most successful strategies for vaccination have been to mimic as closely as possible natural infection, therefore live-attenuated virus vaccines have been widely used. However, such strategy does not function for infections to which natural infection does not lead to long term immunity. Examples of this are parasitic and viral pathogens in which new infections are not prevented by the previous ones.

Most infectious agents enter the body at the mucosal surfaces and mucosal immune responses function as a first line of defense. Protective mucosal immune responses are most effectively induced by mucosal immunization through oral, nasal, rectal, or vaginal routes, but the vast majority of vaccines in use today are administered by intramuscular or subcutaneous injection, which are generally poor inducers of mucosal immunity (Ryan et al. 2001).

Mucosal vaccines that are given orally or deposited directly on the mucosal surfaces face the hosts' defenses as do microbial pathogens: they are diluted in mucosal secretions, captured in mucus gels, attacked by proteases and nucleases, and excluded by epithelial barriers. Thus, relatively large dose of antigens are required and it is impossible to determine exactly what fraction crosses the mucosa. Most soluble non-adherent antigens are taken up in the intestine at low levels and such antigens generally induce tolerance. Thus, several vaccine formulations and delivery strategies have been used to address these challenges and have been revised extensively (Lavelle 2005).

Mucosal vaccines must be particulate or live-vectored, and include immunogenic-conserved forms. These formulations are likely to be most effective when they mimic successful mucosal pathogens in key aspects, efficiently stimulate innate responses, and evoke adaptative responses that are appropriate for the target pathogen. They are ideally multimeric and/or particulate, adhere to mucosal surfaces or even better, adhere to M cells (Ryan et al. 2001; Lavelle 2005).

High doses of antigen are required to induce protective immune responses by the oral route; this requires an

efficient production platform. The initial attempts of using plants as platform production applied conventional nuclear transformation approaches, achieving low levels of expression which limited the dose that could be administered by the oral route. This report where a low level of antigen accumulation was observed followed standard strategies including the use of vectors carrying the gene of interest under the control of a strong promoter such as CaMV35S which is transferred to the plant's genome via *Agrobacterium* (Mason et al. 1992).

Several approaches had been used to increase the protein accumulation in plant tissues. Among these, the optimization of synthetic genes according to codon usage for plants showed to enhance protein levels. The compartmentalization of the target protein through the use of retention signals, such as SEKDEL which direct the protein to endoplasmic reticulum, had a positive effect on the accumulation (Haq et al. 1995).

Later, transplastomic approaches were adopted as a promising strategy that allowed obtaining very high yields giving typical accumulation levels above 10% TSP (Cardi et al. 2010). Promising results have also been obtained by transient expression systems such as Magnifection which combines the viral replication mechanism with the ability of *A. tumefaciens* to transfer DNA to plant cells. These approaches seem to offer the best platform to provide the dose requirements for oral immunization. However, purification steps are necessary in order to rescue the protein of interest to be used for immunization (Gleba et al. 2005, 2007; Santi et al. 2008).

Seed-based expression through conventional nuclear transformation also seems to be a competitive platform since a large volume of recombinant proteins can be produced with increased content by the use of seed-specific promoters as well as localization signals. Additionally, the small size of most seeds allows for the recombinant protein to reach a relatively small volume and constitute a compact biomass in a stable environment being protected from degradation (Zimmermann et al. 2009; Stoger et al. 2005). This system also allows decoupling production of extraction and purification processes due to the storage properties of seeds. Certain seeds also have specific advantages, such as the presence of oil bodies, which can be exploited during the initial purification stages (Stoger et al. 2005). Besides promoters and enhancers, which regulate transcript abundance, subcellular trafficking and targeting of the desired proteins play a crucial role in their accumulation at high levels (Boothe et al. 2010).

Adjuvants

Adjuvants (from "adjuvare": "to help") are compounds that enhance the immunogenicity of vaccine antigens. A recent data suggest that most, if not all, adjuvants enhance T and B cell responses by engaging components of the innate immune system. Benefits of the adjuvant effects include the following: (1) an increase in the response to a vaccine in the general population, (2) an increase of seroconversion rates in populations with reduced responsiveness because of age, (3) the possibility to use smaller doses, (4) the reduction in the number of doses and (5) guiding the type of adaptive response to produce the most effective forms of immunity for each specific pathogen (review in Coffman et al. 2010).

Despite an explosion of knowledge regarding immune function over the recent decades, we remain almost totally reliant for human adjuvants on aluminum-based compounds whose activity was first discovered over 80 years ago. Recent advances in vaccine development and, in particular, the increasing use of recombinant subunit and synthetic vaccines makes the need for improved adjuvants much greater. There is a continuing major need for safe and non-toxic adjuvants, particularly for those capable of strongly boosting cellular immune responses. Despite many advances in immunology, adjuvant development is a priority goal in vaccinology.

A number of carrier proteins have adjuvant effects when epitopes are chemically linked or genetically fused. Besides LT or CT or their B subunits, recently described potential carrier proteins remain to be extensively explored in plant systems. Requirements for efficient carrier proteins are high resistance to low pH and intestinal proteases, and potent immunogenicity by the oral route. For example, the protoxin or toxin Cry1Ac from *Bacillus thurigiensis* might lead to new chimeric proteins to assess the ability to carry unrelated antigens eliciting strong mucosal immune responses (Vázquez et al. 1999; Vázquez-Padrón et al. 1999; Guerrero and Moreno-Fierros 2007).

Plant-derived adjuvants

The adjuvant activity of plant-derived compounds is particularly important in the development of mucosal vaccines. Since pathogens often enter mainly through the mucosal surfaces, new vaccines that elicit protective immunity by means of mucosal administration will be critical. Oral vaccine using plants offer the development of combinatorial approaches where plant producing endogenous adjuvants can be used to produce the recombinant antigen of interest, thus allowing the co-administration of both components, resulting in an enhanced and low cost formulation. This approach would be especially relevant for poor immunogenic proteins as well as for antigens with a low degree of protection against the pathogen.

Plants can produce a wide variety of compounds having adjuvant activity including polysaccharides, saponins,

flavonoids, alkaloids and phenolics. Adjuvant activity has been displayed by many plant-derived compounds (reviewed by Licciardi and Underwood 2010).

It has been reported that the oral administration of phenolic-rich extracts of *Mangifera indica* increases 20 fold the systemic anti-SRBC HA response in mice (Makare et al. 2001). In a study by Nagai and Yamada (1994), oral adjuvanticity was observed by using extracts from the Shoseiryu-to, a Japanese plant-derived medicine which contains the bioactive compound pinellic acid. This effect consisted of enhanced serum anti-influenza HI responses in mice. Moreover, increased anti-influenza virus IgA titres were also higher in the bronchoalveolar lavage fluid (BALF) and nasal wash (Nagai and Yamada 1994, 1998; Nagai et al. 1996).

Saponins from *P. tenuifolia* have also shown to have adjuvant activity producing an increase in serum anti-influenza titres in mice immunized by the in route (Nagai et al. 2001). This effect was attributed to enhanced APC activity and less antigen degradation due to the vaccine formulation leading to sustained exposure to the immune system. It could be proposed that such activity might ultimately provide heightened and prolonged T and/or B-lymphocyte activation resulting in the reported increased antibody response detected.

Similarly, the anti-influenza IgG response of mice orally administered with *Pinellia ternata*-derived extracts was elevated above control mice (Nagai et al. 2002). It was reported that this effect is attributed to pinellic acid, since oral administration of this compound showed the same enhancement on the IgG responses, suggesting that both systemic and mucosal responses can be modulated by this plant-derived adjuvant. These properties of *P. ternata* seem to be associated with indirect activation of B-lymphocytes possibly via APC stimulation similar to the Polygala plant-derived compounds. More recently, Quan et al. (2007) reported that the intranasal co-administration of inactivated influenza virus A and *P. ginseng* on days 0 and 14 significantly enhanced the humoral IgG response to influenza virus, effect likely associated to its high saponin content. Increased lung IgA was also observed 15 days after challenge with influenza virus, this evidence suggests a *P. ginseng* mediated modulation on systemic and mucosal immunity.

Saponins from *Chenopodium quinoa* have shown an adjuvant effect on the cholera toxin (CTX) immunization by the intranasal route (Estrada et al. 1998) which was deducted by increased intestinal serum and lung anti-CTX IgG levels suggesting that these saponins may directly activate the mucosal associated lymphoid system (MALT).

The reported immunomodulatory properties of *Echinacea* have been examined in a number of studies. *Echinacea* is one of the most widely used medicinal plants. A study by Rehman et al. (1999) demonstrated that the oral treatment of rats with an *Echinacea angustifolia* root extract containing a rich source of phenolic compounds (Kuzel et al. 2009) significantly elevated the IgG response to keyhole limpet hemocyanin (KLH) after three immunizations compared to control rats. Similarly, mice orally administered with a combination of *E. purpurea* and *Echinacea pallidae* root extracts increased the anti-SRBC. It was reported that the bioactive properties can differ widely between the various *Echinacea* species, therefore future studies should be based on standardized and well-characterized preparations. Also confirmation of its immune enhancing properties remains to be fully validated in human clinical trials. The vast number of studies using various forms of *Echinacea* and purified compounds make difficult to state conclusive arguments for their use as adjuvants in human vaccines (Pleschka et al. 2009).

Such wide number of plant-derived bioactive molecules with adjuvant properties constitutes promising candidates to be assessed on plant-derived vaccines formulations by co-administration or using the plants able to produce these compounds as expression hosts for the antigen of interest. This trend could lead to new generation of plant-derived vaccines.

Cytokines

Interleukines can play a role as adjuvants influencing the Th1/Th2 balance in the elicited immune response. Some interleukins have been expressed in plants showing to retain their functional properties. This suggests that co-expression of these molecules along with the antigen of interest could lead to adjuvant formulations with the ability of inducing strong immune responses, especially toward predominant Th1 responses which are rarely elicited by soluble subunit vaccines administered by the oral route.

Some cytokines have the potential to serve as adjuvants in the mucosal administration of immunogens. For example, Interleukin 12 (IL-12) is a key cytokine produced by a variety of APCs and promotes cell-mediated Th1 responses, therefore having implications in cancer immunotherapy, treatment of infectious diseases and as adjuvant in vaccine formulations. Mouse IL-12 expression in tomato plants was reported by Gutiérrez-Ortega et al. (2005). This recombinant IL-12 showed to be functional in vitro, determined by interferon-gamma (IFN-gamma) secretion by T cell cultures (Gutiérrez-Ortega et al. 2005). In a later report, functional studies showed that crude extracts of IL-12 administered by the intratracheal route have a specific biological activity reflected as IFN-gamma expression in lungs (Sánchez-Hernández et al. 2010). The expression of a single-chain human interleukin-12 in transgenic tobacco plants has also been reported. The biological activity was

proved by IFN-gamma production assays which revealed comparable levels with respect to the commercially available recombinant IL-12 (Gutiérrez-Ortega et al. 2004). Another human interleukin, hIL-18, was successfully expressed in tobacco confirming its activity by the induction of IFN-gamma on J6-1 cells by plant extracts (Zhang et al. 2003).

Although IL-10 is an anti-inflammatory cytokine, it constitutes another example of a biologically active cytokine produced in plants when administered orally. In 2007, the production of human IL-10 in a low-alkaloid tobacco was described. Its evaluation as contra-inflammatory cytokine reflected a diminution in the severity of colitis in an inflammatory bowel disease animal model (Menassa et al. 2007; Conley et al. 2010). An approach related to cytokine's action was reported by Conrad et al. (2011) whose study consisted in the production of an anti-human TNF single-domain monoclonal antibody (VHH), a major pro-inflammatory cytokine. The aim was to evaluate its potential as TNF antagonist which may have implications on the treatment of inflammatory diseases such as rheumatoid arthritis, Crohn's disease and psoriasis. The fusion protein comprised VHH linked to an elastin-like polypeptide (ELP) and it was produced in tobacco plants. The heterologous protein showed to be biologically active in vivo and it was effective in preventing death caused by septic shock in mice.

These examples opened the doors to the production of functional cytokines that might be expressed in plants which in turn may supplement the plant material containing the antigen of interest. Another approach would be co-expressing both the cytokine and the antigen in the same plant, leading to a single source of the desired formulation. However, expression levels should be regulated to attain the appropriate doses of both components. Studies still need to be carried out to assess the feasibility of these approaches.

Concluding remarks

Plant-based vaccines targeting different diseases through the use of chimeric proteins as immunogens have been of great interest to vaccine developers. Progress over the past decade in the design and evaluation of new broad-protective proteins has demonstrated the feasibility of this technology. However, it is clear that there are a number of new approaches that might improve these candidate vaccines. Although the expression of proteins with the expected activity evaluated in vitro has attracted most of the attention and has been extensively explored, it is clear that strategies for formulation, delivery and evaluation of immunogenicity and even efficiency would profoundly influence the impact of this approach on the vaccine development field. Enhancing specific types of immune responses and generating the multifaceted immune responses that may be needed for challenging diseases is a priority goal.

Along the path of development of new vaccines lies the necessity to a significant investment in manufacturing plants, in human trials which are of great importance and in regulatory aspects before its potential can be exploited. The concept has had many proofs of its efficiency, nonetheless regulatory hurdles will need to be overcome, and industry and public acceptance of the technology are important in order to establish successful products.

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