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Review article

Transgenic carrot plant-made edible vaccines against human infectious diseases

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Abstract

Infectious diseases are becoming a severe public health problem in every part of the world. In response to this, transgenic plants have turned into a new noticeable platform for the production of edible biopharmaceutical proteins, which will be evaluated for their efficiency as edible vaccines to protect against various infectious diseases in humans. Carrot is one plant species that has been used for producing biopharmaceutical proteins. Adjuvants to improve the immune response and bioencapsulation to provide a system for delivering antigens in a way that protects them from the low pH and enzyme digestion inside the gastrointestinal tract have also been developed. The use of transgenic carrot to express the target antigens has been a promising for the production of edible vaccines, because it consists of immunodominant epitopes. The edible vaccines developed so far using carrot could have a crucial role in protecting against various infectious diseases along with the potential to induce both systemic and mucosal immune responses in a mouse model. Herein, we summarize the progress of carrot-based expression systems that have been evaluated for edible vaccine development in the last five years.

Introduction

Various infectious diseases occur worldwide, many of which have been around for hundreds of years, recorded throughout history [1]. Vaccination is the most important to prevent infectious disease in human because of the morbidity and mortality rates have been increasing in many parts of the world. Edible vaccines have been used as an alternative source of protection against some infectious diseases, with benefits that include cost-effectiveness, safety, injection-free, easy to administer without trained personnel, no side effects, high stability, no requirements for special storage conditions, and ease of scaling up production. However, some limitation needs to be improved, including the dosage, vaccine proteins are denatured at the high temperature which could affect the immunogenicity level [2-5]. Most of the research efforts carried out so far have attempted to develop more effective and safer vaccines. The development of edible vaccines is based on the use of recombinant proteins (antigens), which are transgenically in the plant system. Plant-based edible vaccines induce protective immune responses after administration, stimulate innate immunity and memory responses to the target antigen, and use a capable delivery system [6-7].

Molecular farming and genetic engineering technologies have been focused on the development of an effective vaccine that is able to retain appropriate antigenic and immunogenic properties [8-10], which have gained interest. One approach to enhance the immune response and efficient delivery of plant-made edible vaccine is adjuvant such as cholera toxin B subunit (CTB) [11], *Escherichiacoli* heat-labile enterotoxin B subunit (LTB) [12], Rabies virus N protein [13], and human deltaferon (dIFN) [14]. Moreover, the plant cell matrix of bioencapsulation is capable of mediating the delivery of a functional enzyme to the systemic compartment, its high frequency of booster immunization, more realistic immunization approach, ease of administration, multi-dose vaccinations, and the fact that it is very convenient to administer to children make plant-based edible vaccines very promising [15-18]. Of the plants that have, to date, had recombinant proteins successfully expressed in them, some have been used for promising edible vaccine production, including lettuce, potato, tomato, rice, spinach, and maize. They have advanced to phase I clinical trials [19-20], but will have to pass the Food and Drug Administration's (FDA) approval process before being available on the pharmaceutical market. The FDA's views in a genetically modified food which is a combined product was used to evaluate and control of plant-made edible vaccine under good reaping and safety practice regulations [21-22]. In 2009, the first carrot cell was developed a therapeutic protein is verified by FDA to treat Gaucher's

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disease and the product was released to the market in 2012 [23-25]. Otherwise, the utilizations of transgenic carrot plant to produce edible vaccines are being developed.

Carrot (*Daucus carota* L.), which belong to the *Apiece* (Umbelliferae) family, is an important horticultural crop that is commonly consumed in its raw form [26]. Extensive studies have been reported on transgenic carrot plant system due to its high nutritional content, such as vitamin A, β-carotene, carotenoids, lutein, and fiber cellulose [27]. Interestingly, the carotenoids in transgenic carrot could increase lymphocytes, monocytes and immune system in animal models [28]. Currently, several edible products have been made in the transgenic carrot [6], that can induce both systematic and mucosal immune responses (IgA and IgG) in animal models [29]. One breakthrough of the transgenic carrot has been the ability to produce edible vaccines. This review focuses on the progress of transgenic carrot-based edible vaccine development.

Transformation of carrot

The direct and indirect methods are most prevalently used by plant transformations and that methods have been utilized for antigen transformation in the carrot system [26].

The direct-method does not require a vector and uses biolistics (particle bombardment) to transfer the foreign antigen was coated with tungsten or gold as the micro-carrier into the target plant including plant genome, protoplast, and chloroplast [26]. The indirect transformation method is based on the delivery of transgenes by the plant pathogen, *Agrobacterium tumefaciens*, which carries a tumor-inducing plasmid (Ti-plasmid) [30]. Almost all strategies used for gene transformation into the carrot genome have used *Agrobacterium tumefaciens*. This involves DNA being inserted into a T-region of disarmed *Agrobacterium* Ti plasmid, achieved by co-culturing it with plant cells under genes encoding the plant hormones (auxins and cytokinins) [30-31]. The benefits of this method include, stability, simplicity, cost effectiveness, and the ability of a large fragment and multiple genes being introduced into the plant cell [30-32].

In order to obtain a high level of recombinant proteins were combined with signal sequence peptide, and the selectable marker gene under the control of the 35S promoter of the cauliflower mosaic virus (CaMV) that is promotor used in plants, before being expressed in the carrot [27]. The antigens were fused to signal peptides such as Ser-Glu-Lys-Asp-Glu-Leu (SEKDEL) [16, 29, 33] and His-Asp-Glu-Leu (HDEL) at the C-terminus [34]. They were expressed in the endoplasmic reticulum (ER) where they are highly useful in increasing protein stabilization (without the protein glycosylation process) and enhancing protein expression [33-35].

The transgenic carrot plant based edible vaccines have rarely combined with signal peptide, which could be performed protein accumulation which specific to targeting and some of them was successfully improved the yield accumulation (Table 1). The selectable marker genes used for carrot transformation were *nptII* gene and *bar* gene. They are classical marker genes that have been used to select the transformants plants for kanamycin resistance (neomycine phosphotransferase) and resistance to the herbicide Basta(phosphinothricinacetyl transferase), respectively [36]. Basta has been successfully used to select the transformed carrot cells in liquid medium and for the regeneration of adult transformed plants by Rojas-Anaya *et al* [13].

Table1. Parts of transgenic carrot used for different vaccines

Infectious Disease	Promoter	Protein Signal Peptide	Target of Expression System	Expression Level	References
Hepatitis B virus	CaMV35S	HDEL	Leaves	36 ng/g fresh weight	[34]
Human Immunodeficiency Virus type 1 (HIV-1)	CaMV35S	SEKDEL	Taproot	62 ng /g fresh weight	[16,29]
Helicobacter pylori	CaMV35S	n.d.	Root	25 µg /g fresh weight	[37]
Chlamydia trachomatis	CaMV35S	n.d.	Cell suspension	3% TSP	[17]
Cholera and ETEC diarrhea	CaMV35S	n.d.	Callus	3 µg of protein /g of tap root	[5,38]
Mycobacterium tuberculosis	CaMV35S	n.d.	Taproots	28.140 μg of TSP	[14]
Rabies virus	CaMV35S	n.d.	Taproots	0.2 to 1.4% TSP	[13]
Porcine cysticercosis	CaMV35S	n.d.	Callus	14 µg/g dry weight	[39]

TSP = Total soluble protein, n.d. = not determined

Mucosal immune response and oral tolerance

The gut-associated lymphoid tissue (GALT) is an important organ of the immune system, and it is the preliminary organ to be exposed to antigens [40]. GALT has a vital role in creating a tolerogenic environment, which tolerance induction occurs upon suppression of the immune system [41]. Tolerance induction is dependent on the dose of antigen (high dose) and the number of regulatory T-cells (Tregs) (low dose) [40]. While the Peyer's patches (PP) are the inductive sites in the gut immune system [42], which have been found to be the site of oral tolerance induction. They are also the largest lymph nodes in the body, and are essential for mucosal tolerance. Peyer's patches consists of macroscopic lymphoid tissue, assembled in the submucosa along the length of the small intestine, along with mesenteric lymph nodes (MLNs) [40]. Additionally, dendritic cells (DCs) in the MLNs that recognize antigens are important components of the intestinal immune system involved in the induction of oral tolerance [43].

Adjuvants and bioencapsulation

In order to produce a successful edible vaccine that can be effectively administrated, the antigens need to be fused to an adjuvant under T lymphocyte regulation; this has a crucial role in regulation of the immune response by T helper lymphocytes, which will respond to antigens presented by antigen presenting cells (APC). The antigenis taken up by Microfold cells (M-cells) and an immune response against the target infectious disease is initiated. Interleukin-12 (IL-12) enhanced Th1-immune responses and promotes the secretion of immunoglobulin G (IgG) into the blood and immunoglobulin A (IgA) from B lymphocytes within mucosal lymphoid tissue [44].

Adjuvants enhance the immune response to a specific antigen and induce immunonogical memory of antigen vaccines [45]. They also have the potential to stimulate both systemic and mucosal immune responses [17]. The carrot expression system has been used to study various adjuvants such as cholera toxin B subunit (CTB) [11], *Escherichiacoli* heat-labile enterotoxin B subunit (LTB) [12], rabies virus N protein [13] and human deltaferon (dIFN) [14]. CTB is an immunogenic carrier, which act as an adjuvant to antigen vaccines and stimulates antibody production. In addition, it enhances the immunogenicity of the antigen in human and animals [46]. LTB is a non-toxic potent immunogen [38], that has been used as vaccine adjuvant to induce a strong immune response and has been approved for use in clinic application in humans [47]. Rabies virus N protein is a major antigen capable of inducing virus-specific helper T cells and antibodies [48]. It can be co-expressed with antigen of interest for particular vaccines and has been shown to enhance the body's immune response to vaccine[49]. The *dIFN* gene is encoded deltaferon, a recombinant analogue of displays the immunomodulatory properties of an adjuvant [50]. It has been used to increase the mucosal immunogenicity of antigens and to induce both humoral and cell-mediated immune responses in mouse models [14].

Bioencapsulation of the immunogen within the plant cell matrix [47], such as pectin and cellulosic cell walls, has been useful in maintaining vaccine stability, and protecting against enzyme digestion and low pH in the stomach. Bioencapsulation has been shown to induce high immunogenicity, and it allows the antigen protein to be released into the gut lumen and other mucosal tissues [5,13,14,37,51-55]. The plant cell wall is similar to liposomes and microcapsules, so in the development of edible vaccines, it could offer oral priming [30].

The development of carrot plant-made edible vaccines

Carrot can be manipulated to produce novel edible vaccines, and new sources of biopharmaceuticals. Research efforts have attempted to deal with the problem with emerging infectious diseases considering the need for low cost vaccines, high production, and the need for availability in developing countries. Several carrot plants-based vaccines that have been developed in the last five years are described below.

***Helicobacter pylori* vaccine**

Helicobacter pylori is a species of bacteria that colonizes the stomach, and causing chronic gastritis, peptic ulcers. It has been linked to gastric cancer and mucosal-associated lymphoid tissue lymphoma [56]. Ure B, a *H. pylori* -protective antigen, could prevent bacterial infection. The Ure B recombinant protein was combined with cholera toxin B subunit (CTB) and then transformed into the carrot plant genome using the CaMV35 S promoter, *Agrobacterium tumefaciens* strain LBA4404. Finally, the transformed plant cells were selected using kanamycin. The recombinant protein was successfully produced and accumulated in the root with a yield of up to 25 µg/g of fresh weight. The extracted Ure B protein was fed in mouse models at a dose 5 g transgenic carrot roots and was shown to the effectively induce mucosal immune response and a highly specific antibody titer [37].

***Chlamydia trachomatis* vaccine**

Chlamydia trachomatis is a gram negative bacterium belonging to the genus *Chlamydia*. It is an infectious disease, transmitted via sexual contact. The infection-occurs in men and women and has been linked to pelvic inflammatory disease, infertility, ectopic pregnancy in women, and epididymitis and orchitis in men [57]. *Chlamydia trachomatis*antigenic-epitopes for helper and cytotoxic T lymphocytes, contain a major outer membrane protein (MOMP), which consists of VS2 and

VS4 loops that could decrease hydrophobicity, and the viable domain regions (VDS) of the MOMP. The expression level of chimeric MOMP was shown to be -up to 3% of the total soluble protein in carrot cells when driven by the CaMV 35S promoter via *Agrobacterium tumefaciens*. The transformed carrot cells were selected on herbicide Basta. To date, however, no *Chlamydia trachomatis* protein has been used for oral immunization in animal models [17].

Cholera and ETEC diarrhea vaccine

Diarrheal diseases caused by Vibrio cholerae and enterotoxigenic *Escherichia coli* (ETEC) are infectious diseases, caused by contaminated drinking water in tropical climates, and have become global health problems. The immunodominant epitopes of ETEC was chosen as the vaccine target antigen and the adjuvant chosen was *Escherichiacoli* heat-labile enterotoxin B subunit (LTB). The LTB gene was transformed into carrot cells using the *Agrobacterium tumefaciens* strain LBA4404 and selected on kanamycin. An immune response in mice was successfully elicited using 10 µg of carrot-derived LTB. This dose also elicited a significant serum anti-LTB antibody level and showed specific IgG antibody responses in the intestine. Even though IgA antibody levels were detected in the large intestine of mice, levels of transgenic carrot expressed LTB protein was lower than that of pure recombinant LTB administration. However, the level obtained was high enough to protect mice against the cholera toxin, and a Th2 response was induced by IgG and IgA via oral administration [5].

***Mycobacterium tuberculosis* vaccine**

Tuberculosis is a chronic infectious disease caused by the *bacterium, Mycobacterium tuberculosis* (*M. tuberculosis*), which is one of the main global health problems in humans. Several protein subunits of *M. tuberculosis* vaccines have been considered, including those that are secreted from the bacterial cell wall. Tuberculosis vaccine expression in plant cells consists of antigen to activate $CD4^+$ helper and $CD8^+$ cytotoxic T lymphocytes and induce protective immunity. The CFP10-ESAT6 and human deltaferon (dIFN) fusion genes, along with the CaMV 35S promoter were transferred into the carrot genome using *Agrobacterium tumefaciens*. Mice were immunized by oral administration at a dose 5 gtransgenic carrot. The antigens could induce both cell-mediated and humoral immunity and could also reduce the cytotoxic effect in a mouse model [14,52].

Human immunodeficiency virus type 1 (HIV-1) vaccine

Human immunodeficiency virus type 1 (HIV-1) is a serious viral disease, that causes a long-life infection with no cure to date. There is also no vaccine available, so there has been an extensive research effort to produce a subunit HIV-1 vaccine. One such vaccine that retained the antigen epitope was considered for its safety aspect. The HIV-1 subtype C p24 protein was used to produce an oral vaccine in Carrot and Cress (*Arabidopsis thaliana*). The SEKDL amino acid sequence was combined with the p24 protein and used to produce the antigenic protein in the ER under the control of the CaMV 35S promoter. Positive transformants were selected on herbicide Basta. The product of recombinant the p24_SEKDEL protein was produced at a yield of 62 ng p24/g fresh weight in carrot cells, which was lower than expression in cress (139 ng p24 protein/g fresh weight). The protein produced was highly stable during cultivation and accumulated in the endoplasmic reticulum (ER) [29]. Mice that were immunized by oral administration at a dose 720 ng of p24 antigen (40 g of fresh weight transgenic carrot), followed by a booster intramuscular injection, were found to have antigen specific IgG antibodies that induced humoral immune response and induced neutralizing antibodies in patients [16,29].

Rabies virus vaccine

Rabies virus is a deadly infectious viral disease that is a global public health problem. There is no cure for rabies after symptoms have started, meaning it have a very highly mortality rate, with an estimated 50,000 human deaths in annually worldwide, 90% of which occur in developing countries [58]. The rabies virus glycoprotein (rabies G protein) antigen was designed from using the cytoplasm domain, transmembrane, and actodomain, which could protect against viral infection and induce neutralizing antibodies. Rabies G protein was expressed in carrot cell under the control of the CaMV 35S promoter using the microprojectile bombardment method. The transformed cells were then selected for using herbicide Basta. Mice were immunized with 2 g of raw carrot plus 50 µg of rabies virus N protein by the oral route and were shown to have developed protective rabies antibodies. Rabies virus N protein was used as the adjuvant to prevent enzyme digestion in the gastrointestinal tract and enhance immunogenicity [13].

Porcine cysticercosis vaccine

Porcine cysticersosisis is caused by the larvae of the *Taenia solium* (*T. solium*) in pigs. *T. solium* is transmitted via the oral-fecal route to humans if infected under-cooked pork is consumed. It can cause neurocysticercosis in the human central nervous system (CNS) and is associated with chronic headache, blindness, seizures, hydrocephalus, meningitis, and dementia [59]. The recombinant GST-HP6/TSOL18 gene of the anti- *T. solium cysticercosis* antigen was expressed in carrot cells under the control CaMV 35S promoter and using *Agrobacterium tumefaciens* strain GV3101. The electroporation method was used of the transformation,

and positive cells were selected using kanamycin. Protein expression levels reached approximately 14 µg/g dry-weight of carrot calli. Mice immunized with 700 ng of recombinant HP6/TSOL18 antigen showed the potential to be protected against the cysticercosis infection. The mice showed a significant reduction in parasite burden, increased high levels of IgG serum antibodies, and increased mucosal IgA antibodies in the intestines [39].

Conclusion

Although there is no transgenic carrot plant-made edible vaccine, which has been approved in clinical trials. But the advances in the production of edible vaccine in the carrot system could be helped to protect against infectious diseases in animal models, and eventually humans. However, there are some limitations to using the carrot system has a low protein expression. Despite this, edible vaccine development and production is still urgently needed. The mechanism of plant bioencapsulation developed, using the plant cell wall, has been shown to be effective, with both mucosal and systemic immune responses being elicited. At present we know that the carrot system is a possibility for the alternative production of edible vaccines; however, more studies are needed to focus on the challenges of carrot-made edible vaccines for infectious diseases, such as low production costs and ease of delivery.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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