Edible Vaccines

12

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Abstract

In recent years edible vaccine emerged as a new concept developed by biotechnologists. Edible vaccines are subunit vaccines where the selected genes are introduced into the plants and the transgenic plant is then induced to manufacture the encoded protein. Foods under such application include potato, banana, lettuce, corn, soybean, rice, and legumes. They are easy to administer, easy to store and readily acceptable delivery system for different age group patients yet cost effective. Edible vaccines present exciting possibilities for significantly reducing various diseases such as measles, hepatitis B, cholera, diarrhea, etc., mainly in developing countries. However, various technical and regulatory challenges need to overcome in the path of this emerging vaccine technology to make edible vaccine more efficient and applicable. This chapter attempts to discuss key aspects of edible vaccines like host plants, production, mechanism of action, advantages and limitations, applications, and different regulatory issues concerned to edible vaccines.

12.1 Introduction

Vaccine is a biological preparation intended to produce immunity to a disease by stimulating the production of antibodies. Dead or attenuated organisms or purified products derived from them are generally used to produce various vaccines. Over the past decade, scientific advances in genetics, molecular biology, and plant biotechnology have improved the understanding of many infectious diseases and led to the development of vaccination programs. The most common method of administering vaccines is by injection but some are given by mouth or nasal spray. Though immunization is the safest method to combat the diseases worldwide but there are many constraints regarding its mode of production, distribution, delivery, cost, and lack of enough research. Hence it is desirable to look for an effectual and powerful yet cost effective, easy for storage and distribution yet safe method of immunization. It should also be readily

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acceptable to all sociocultural groups around the globe. Research underway is dedicated to solving these problems by finding ways to produce edible vaccines in the form of transgenic plants which have been investigated as an alternative means to produce and deliver vaccine.

Edible vaccines are called by several alternative names such as food vaccines, oral vaccines, subunit vaccines, and green vaccines. They seem to be a viable alternative especially for the poor and developing countries. They have come up as great boon in medicinal science for which biotechnologists should be given all credit. The concept of edible vaccines lies in converting the edible food into potential vaccines to prevent infectious diseases. It involves introduction of selected desired genes into plants and then inducing these altered plants to manufacture the encoded proteins. It has also found application in prevention of autoimmune diseases, birth control, cancer therapy, etc. Edible vaccines are currently being developed for a number of human and animal diseases. This new technology hopefully will contribute positively toward the global vaccine programs and have a dramatic impact on health care in developing countries.

12.2 Historical Background

Many people in developing countries do not have access to the vaccines they need, as the traditional vaccines are costly and require skilled medical people for administration and are less effective in inducing mucosal immune response. It was these needs which inspired Hiatt et al. (1989) who attempted to produce antibodies in plants which could serve the purpose of passive immunization. The first report of edible vaccine (a surface protein from *Streptococcus*) in tobacco, at 0.02 % of total leaf protein level, appeared in 1990 in the form of a patent application published under the international patent cooperation treaty. By conceiving the idea of edible vaccine Dr. Charles Arntzen tried to realize it (Arntzen 1997). In 1992, Arntzen and coworkers introduced the concept of transgenic plants as a production and delivery system for subunit vaccines in which edible tissues of transgenic crop plants were used (Mor et al. 1998). They found that this concept could overcome the limitations of traditional vaccines, thereby triggering the research on edible vaccine. In 1990s, *Streptococcus mutans* surface protein antigen A was expressed for the first time in tobacco. The same group also pioneered the field with work on hepatitis B and heat-labile toxin, B subunit in tobacco plants and potato tubers. In the same year, the successful expression of hepatitis B surface antigen (HBsAg) in tobacco plants was also achieved (Mason et al. 1992). To prove that plant-derived HBsAg could stimulate mucosal immune responses *via* oral route, potato tubers were used as an expression system and were optimized to increase the accumulation of the protein in plant tubers (Richter et al. 2000).

Parallel to the evaluation of plant-derived HBsAg, Mason and Arntzen explored plant expression of other vaccine candidates including the labile toxin B subunit (LT-B) of enterotoxigenic *Escherichia coli* (ETEC) and the capsid protein of Norwalk virus. The plant-derived proteins correctly assembled into functional oligomers that could elicit the expected immune responses when given orally to animals (Mason et al. 1998).

In 1998 a new era was opened in vaccine delivery when researchers supported by the National Institute of allergy and infectious diseases (NIAID) have shown for the first time that an edible vaccine can safely generate significant immune responses in people. The report by collaborators from the University of Maryland in Baltimore, the Boyce Thompson Institute for Plant Research in Ithaca, N.Y., and Tulane University in New Orleans appeared in the May issue of Nature Medicine. According to the then Director of NIAID "Edible vaccines offer exciting possibilities for significantly reducing the burden of diseases like hepatitis and diarrhea, particularly in the developing world where storing and administering vaccines are often major problems," Mor et al. (1999) also discussed the rapid increase of research in the edible-vaccine field and pointed out that plants

could be used to create multicomponent vaccines that can protect against several pathogens at once. This is an aspect of the edible-vaccine approach that further strengthens its impact. Later, in 2003 Sala and research group reported that proteins produced in these plants induced the mucosal immune response which was the main aim behind this concept.

Research into edible vaccine is still at a very early stage and scientists have a long way to go before it will become a major part of immunization program world wide.

12.3 Choice of Host Plant for Edible Vaccine

To date, many plant species have been used for vaccine production. The choice of the plant species is important. An edible, palatable plant is necessary if the vaccine is planned for raw consumption. In case of vaccine for animal use, the plant should preferentially be selected among those consumed as normal component of the animal's diet. Some food vehicles are discussed below:

12.3.1 Tobacco

The concept of edible vaccine got impetus after Arntzen and coworkers expressed HBsAg in tobacco. The first edible vaccine was produced in tobacco in 1990 in which 0.02 % recombinant protein (a surface protein from *Streptococcus*) of the total soluble leaf proteins was found. It appeared in the form of a patent application published under the International Patent Cooperation Treaty. Transgenic tobacco is successfully engineered for the production of edible vaccines against hepatitis B antigen using 's' gene of hepatitis B virus (HBV). The optimum level of recombinant protein was obtained in leaves and seeds. Since acute watery diarrhea is caused by enterotoxigenic *E. coli* and Vibrio cholerae that colonize the small intestine and produce one or more enterotoxin, an attempt was made toward the production of edible vaccine by expressing heat-labile enterotoxin (LT-B) in tobacco. Besides, antibodies against dental caries, expressed in tobacco, are already in preclinical human trials. Italian researchers have now developed an immunologically active, cost-efficient vaccine against human papilloma viruses (HPV). HPV are the causative agents for cervical cancer, and are also involved in skin, head, and neck tumors. Cervical cancer is one of the main causes of cancer-related deaths.

12.3.2 Potato

Genetically modified potatoes are also a viable option and seem to be the desired vector. Many of the first edible vaccines were synthesized in potato plants. The transgenic potatoes were developed and grown by Arntzen and Mason and their research group at the Boyce Thompson Institute for Plant Research, Cornell University. Previously, NIAID supported *in vitro* and preclinical studies by John Clements and colleagues at Tulane University School of Medicine, in which 14 volunteers ate bite-sized pieces of raw potato that had been genetically engineered to produce part of the toxin secreted by *E. coli* causing diarrhea. The investigators periodically collected blood and stool samples from the volunteers to evaluate the vaccine's ability to stimulate both systemic and intestinal immune responses. Ten of the 11 volunteers (91 %) who ingested the transgenic potatoes had fourfold rise

in serum antibodies at some point after immunization, and 6 of the 11 (55 %) also showed fourfold mount in intestinal antibodies. The potatoes were well tolerated and no one experienced serious adverse side effects. Vaccine development has successfully tested a potato-based vaccine to combat the Norwalk Virus, which is spread by contaminated food and water. The virus causes severe abdominal pain and diarrhea.

A research team led by William Langridge of the Loma Linda University in California has reported that transgenic potatoes engineered with a cholera antigen, CTB can effectively immunize mice. Mice fed transgenic potatoes produce cholera-specific antibodies in their serum and intestine; IgA and IgG antibodies reach their highest levels after the fourth feeding. In yet another experiment genetically engineered potatoes containing a hepatitis B vaccine have successfully boosted immunity in their first human trials.

Attempts have also been made to boil the potatoes as raw potatoes are not very appetizing but unfortunately the cooking process breaks down about 50 % of the proteins in the vaccine. While some proteins are more tolerant to heat, for most proteins it will be necessary to amplify the amount of protein in the engineered foods if they are to be cooked before consumption.

12.3.3 Tomato

Tomatoes are an excellent candidate because they are easy to manipulate genetically and new crops can be grown quickly. Moreover, they are palatable and can be eaten raw. While tomatoes do not grow well in the regions in which the edible vaccines are most needed, the engineered tomatoes can be dried or made into a paste to facilitate their delivery.

The anti-malaria edible vaccines in different transgenic tomato plants expressing antigenic type(s) have been proposed by Chowdhury and Bagasara in 2007. They hypothesized that immunizing individuals against 2–3 antigens and against each stage of the life cycle of the multistage parasites would be an efficient, inexpensive and safe way of vaccination. Tomatoes with varying sizes, shapes, and colors carrying different antigens would make the vaccines easily identifiable by lay individuals.

Tomatoes serve as an ideal candidate for the HIV antigen because they unlike other transgenic plants that carry the protein, are edible and immune to any thermal process, which help to retain their healing capabilities. Scientists have claimed that tomatoes could be used as a vaccine against Alzheimer's disease. The work is in progress to genetically modify the fruit to create an edible vaccine that fires up the immune system to tackle the disease by attacking the toxic beta-amyloid protein that destroys vital connections between brain cells, causing Alzheimer's.

Researchers have engineered tomato plants (*Lycopersicon esculentum* Mill var. UC82b) to express a gene for the glycoprotein (G-protein), that coats the outer surface of the rabies virus. The recombinant constructs contained the G-protein gene from the ERA strain of rabies virus, including the signal peptide, under the control of the 35S promoter of cauliflower mosaic virus (CaMV).

12.3.4 Banana

A common fruit—the banana—is currently being considered as a potential vehicle for vaccines against serious as well as too common diseases. The advantage of bananas is that they

can be eaten raw as compared to potatoes or rice that need to be cooked and can also be consumed in a pure form. Furthermore, children tend to like banana and the plants grow well in the tropical areas in which the vaccines are needed the most. Hence, the research is leaning toward the use of banana as the vector since a large number of third-world countries, who would benefit the most from edible vaccines have tropical climates. On the negative side, a new crop of banana plants takes about 12 months to bear fruit. After fruiting, the plants are cut down and a new crop of vaccine-bearing plants must be planted.

Researchers have also developed bananas that deliver a vaccine for HBV. The banana vaccine is expected to cost just 2 cents a dose, as compared to the $125 for the currently available injectable vaccine.

12.3.5 Maize

Maize has also been used as a vector for various edible vaccines. Egyptian scientists have genetically engineered the maize plants to produce a protein known as HbsAg which elicits an immune response against the hepatitis B virus and could be used as a vaccine. If human trials are successful more than 2 billion people infected with hepatitis B, and about 350 million of these at high risk of serious illness and death from liver damage and liver cancer would be benefited.

Researches are in offing at Iowa State University with the aim to allow pigs and humans to get a flu vaccination simply by eating corn or corn products. It is quite likely that corn vaccine would work in humans when they eat corn or even corn flakes, corn chips, tortillas, or anything that contains corn.

Genetically modified maize could provide protection to chickens against a highly contagious and fatal viral disease affecting most species of birds. Mexican researcher Octavio Guerrero-Andrade and his colleagues at the Centre for Research and Advanced Studies in Guanajuato, Central Mexico, genetically modified maize to create an edible vaccine against Newcastle disease virus (NDV). They inserted a gene from the NDV, a major killer of poultry in developing countries, into the maize DNA and found antibodies against the virus in chickens that ate the genetically modified maize. One pig vaccine has also been produced in corn successfully.

Efforts are being made by US company ProdiGene to genetically modify maize to contain a key protein found on the surface of the monkey form of HIV. According to US National Institute of Health this development brings an edible, more effective, HIV vaccine for people a step closer.

Transgenic maize expressing the rabies virus glycoprotein (G) of the Vnukovo strain has also been produced using ubiquitin maize promoter fused to the whole coding region of the rabies virus G gene, and a constitutive promoter from CaMV. Maize embryogenic callus were transformed with the above construct by biolistics. Regenerated maize plants were recovered and grown in a greenhouse. The amount of G-protein detected in the grains was approximately 1 % of the total soluble plant protein.

12.3.6 Rice

Rice is another potential crop which has been used for developing vaccines. It offers several advantages over traditional vaccines; it does not require refrigeration. In fact, the rice proved just as potent after 18 months of storage at room temperature and the vaccine did not dissolve when exposed to stomach acids. In an attempt, predominant T cell epitope peptides, which were derived from Japanese cedar pollen allergens, were specifically expressed in rice seeds and delivered to the mucosal immune system (MIS); the development of an allergic immune response of the allergen-specific Th2 cell was suppressed. Furthermore, not only the specific IgE production and release of histamine from mast cells were suppressed, but the inflammatory symptoms of pollinosis, such as sneezing, were also suppressed. These results suggest the feasibility of using an oral immunotherapy agent derived from transgenic plants that accumulate T cell epitope peptides of allergens for allergy treatment.

The transfer of genetic material from the microbe responsible for producing cholera toxin into a rice plant has been achieved. The plants produced the toxin and when the rice grains were fed to mice they provoked immunity from the diarrhea-causing bacterium.

12.3.7 Spinach

Genetically modified spinach has also been considered for the development of edible vaccine. Spinach is being investigated as a plant-derived, edible vehicle for anthrax vaccine, as well as a vehicle for the HIV-1 Tat protein (a prospective vaccine candidate). In an experiment a fragment of protective antigen (PA) that represents most of the receptor-binding domain was expressed as a translational fusion with a capsid protein on the outer surface of tobacco mosaic virus, and spinach was inoculated with the recombinant virus. The plant-expressed PA is highly immunogenic in laboratory animals.

Among other food crops with potential to be developed as edible vaccine; sweet potato, peanuts, lettuce, watermelon, and carrots are on the top priority. The development of plant-based vaccines to protect against many other diseases, such as HIV-1, hepatitis B, rabies, and non-Hodgkin's lymphoma are ongoing throughout the globe using one of these edible plants.

The advantages and disadvantages of various plant host systems are given in Table 12.1.

12.4 Advantages

Conventional subunit vaccines are expensive and technology-intensive, need purification, require refrigeration, and produce poor mucosal response. In contrast, edible vaccines would

Table 12.1 Features of different plant host systems

Plant	Advantages	Disadvantages
Tobacco	Facile and efficient transformation system; abundant material for protein characterization	Toxic alkaloids incompatible with oral delivery; potential for outcrossing in field
Banana	Cultivated widely in developing countries where vaccines are needed; eaten raw by infants and adults; clonally propagated; low potential for outcrossing in field; once established, plentiful and inexpensive fruits are available on a 10–12 month cycle	Inefficient transformation system; little data available on gene expression, especially for fruit specific promoters; high cultivation space requirement; very expensive in greenhouse
Potato	Facile and efficient transformation system; tuber is edible raw though not palatable; tuber-specific promoters available; microtuber production for quick assay; clonally propagated, low potential for outcrossing in field; Industrial tuber processing well established	Relatively low tuber protein content; unpalatable in raw form; cooking might cause denaturation and poor immunogenicity of vaccine
Tomato	Relatively efficient transformation system; fruit is edible raw; fruit specific promoters available; crossing possible to stack antigen genes; industrial greenhouse culture and industrial fruit processing well established	Relatively low fruit protein content; acidic fruit may be incompatible with some antigens or for delivery to infants; no *in vitro* system to test fruit expression
Legumes	Production technology widely established; high protein content in seeds; stable protein in stored seeds; well suited for animal vaccines; industrial seed processing well established	Inefficient transformation systems; heating or cooking for human use might cause denaturation and poor immunogenicity of vaccine; potential for outcrossing in field for some species
Alfalfa	Relatively efficient transformation system; high protein content in leaves; leaves edible uncooked	Potential for outcrossing in field; deep root system problematic for cleaning field

(Mason et al. 2002)

enhance compliance, especially in children, and because of oral administration would eliminate the need for trained medical personnel. Their production is highly efficient and can be easily scaled up. For example, hepatitis B antigen required to vaccinate whole of China annually, could be grown on a 40-acre plot and all babies in the world each year on just 200 acres of land. They are cheaper, sidestepping demands for purification (single dose of hepatitis B vaccine would cost approximately 0.43 cents), grown locally using standard methods and do not require capital-intensive pharmaceutical manufacturing facilities. Mass-indefinite production would also decrease dependence on foreign supply. Fear of contamination with animal viruses—like the mad cow disease, which is a threat in vaccines manufactured from cultured mammalian cells, is eliminated as plant viruses do not infect humans.

Edible vaccines activate both mucosal and systemic immunity, as they come in contact with the digestive tract lining which is not possible with subunit vaccines which provide poor mucosal response. This dual effect of edible vaccines provides first-line defense against pathogens invading through mucosa, such as *Mycobacterium tuberculosis* and agents causing diarrhea, pneumonia, STDs, HIV, etc.

The specific advantages are stated below:

1. Edible means of administration.
2. No need of medical personnel and syringes.
3. Sterile injection conditions are no more required.
4. Economical in mass production by breeding compared to an animal system.
5. Easy for administration and transportation.
6. Effective maintenance of vaccine activity by controlling the temperature in plant cultivation.
7. Therapeutic proteins are free of pathogens and toxins.
8. Storage near the site of use.
9. Heat stable, thus eliminating the need of refrigeration.

10. Antigen protection through bioencapsulation.
11. Subunit vaccine (not attenuated vaccine) means improved safety.
12. Seroconversion in the presence of maternal antibodies.
13. Generation of systemic and mucosal immunity.
14. Enhanced compliance (especially in children).
15. Delivery of multiple antigens.
16. Integration with other vaccine approaches.
17. Plant-derived antigens assemble spontaneously into oligomers and into virus like particles.
18. No serious side effect problems have been noticed until now.
19. Reduced risk of anaphylactic side effects from edible vaccine over injection system is one benefit reported by the Bio-Medicine.org. They reported that the edible vaccine carries only part of the allergen compared to injection methods which reduce anaphylactic risk.
20. Administration of edible vaccines to mothers to immunize the *fetus*-in utero by transplacental transfer of maternal antibodies or the infant through breast milk. Edible vaccines have a potential role in protecting infants against diseases like group-B *Streptococcus*, respiratory syncytial virus (RSV), etc., which is under investigation.
21. Edible vaccines would also be suitable against neglected/less common diseases like dengue, hookworm, rabies, etc. They may be integrated with other vaccine approaches and multiple antigens may also be delivered.

12.5 Limitations and Challenges

With advancement come many hurdles and problems, so is true for edible vaccines. Like, one could develop immunotolerance to the vaccine peptide or protein, though a little research has been done on it. One of the key goals of the edible-vaccine pioneers was to reduce immunization costs but later many limitations were reported as given below:

1. Consistency of dosage from fruit to fruit, plant to plant, lot to lot, and generation to generation is not similar.
2. Stability of vaccine in fruit is not known.
3. Evaluation of dosage requirement is tedious.
4. Selection of best plant is difficult.
5. Certain foods like potatoes are generally not eaten raw and cooking the food might weaken the medicine present in it.
6. Not convenient for infants as they might spit it, eat a part or eat it all, and throw it up later. Concentrating the vaccine into a teaspoon of baby food may be more practical than administering it in a whole fruit.
7. There is always possibility of sideeffects due to the interaction between the vaccine and the vehicle.
8. People could ingest too much of the vaccine, which could be toxic, or too little, which could lead to disease outbreaks among populations believed to be immune.
9. A concern with oral vaccines is the degradation of protein components in the stomach due to low pH and gastric enzymes. However, the degradation can be compensated by repeating the exposure of the antigen until immunological tolerance is accomplished (Mason et al. 2002).
10. Potential risk of spreading the disease by edible vaccine delivery is a concern of many. Potential contamination of the oral delivery system is a possible danger.

Foreign proteins in plants accumulate in low amounts (0.01–2 % of total protein) and are less immunogenic, therefore the oral dose far exceeds the intranasal/parenteral dose. For example oral hepatitis B dose is 10–100 times the parenteral dose and 100 g potato expressing B subunit of labile toxin of ETEC (LT-B) is required in three different doses to be immunogenic. Attempts at boosting the amount of antigens often lead to stunted growth of plants and reduced tuber/fruit formation as too much mRNA from the transgene causes gene silencing in plant genome.

Techniques to overcome these limitations are given below:

1. Optimization of coding sequence of bacterial/viral genes for expression as plant nuclear genes
2. Expression in plasmids
3. Plant viruses expressing foreign genes
4. Coat-protein fusions
5. Viral-assisted expression in transgenic plants
6. Promoter elements of bean yellow dwarf virus with reporter genes GUS (β -glucuronidase) and green fluorescent protein (GFP), substituted later with target antigen genes.
7. Antigen genes may be linked with regulatory elements which switch on the genes more readily or do so only at selected times (after the plant is nearly fully grown) or only in its edible regions. Exposure to some outside activator molecule may also be tried.

12.6 Side Effects

Development of edible vaccines is a possible high-volume, low-cost delivery system for third-world countries to fight against fatal maladies like AIDS, hepatitis, and diarrhea. Researches by the NIAID and the University of Maryland showed no significant side effects in a small study using genetically engineered potatoes to make toxin of the *E. coli*, a diarrhea-causing bacterium. Volunteers reported no serious adverse reactions to genetically altered potatoes used to deliver edible vaccine toxin, according to the National Institutes of health (NIH). The NIH reported that 10–11 volunteers who ate the raw potato bites developed four times the antibodies against *E. coli* without obvious side effects.

Long-term reactions to edible vaccines are yet to be determined and possible delayed reactions have not yet been discovered. An organized large scale study is required before edible vaccines are put into large scale production.

12.7 Production of Edible Vaccines

Creating edible vaccines involves the genetic engineering approach for the introduction of selected desired genes into plants and then inducing these altered plants to manufacture the encoded proteins. This process is known as "transformation" and the plants altered with new characteristics are called "transgenic plants". Like conventional subunit vaccines, edible vaccines are composed of antigenic proteins and are devoid of genes responsible for pathogenicity. Thus, they have no way of establishing infection, assuring its safety, especially in immune-compromised patients.

The gene which codes the active antigenic protein is first isolated from the pathogen and is incorporated in a suitable "gene vehicle". This gene vehicle is integrated into the genome of the plant and is allowed to express the corresponding antigen. Then these plant parts are fed to animals and humans to run their course.

Two main strategies are used for the production of candidate vaccine antigen in plant tissues (Fig. 12.1 a, b).

1. Stable genomic integration: This is the most popular method used for the published edible vaccine clinical trials to date. Under this method the genetic line is produced that can be propagated either by vegetative (stem cuttings) or sexual (seeds) reproduction methods. The stable expression strategy provides an opportunity to introduce more than one gene for possible multicomponent vaccine production. Furthermore, the choice of genetic regulatory elements allows organ and tissue-specific expression of foreign antigens. Stable transformation causes the desired gene to be incorporated either in nucleus or chloroplast. *Agrobacterium* mediated gene transfer is used for transforming the plants in which the gene is integrated in nucleus. Besides, direct delivery of DNA into the tissue can also be applied, biolistic being the most popular method. However, chloroplast

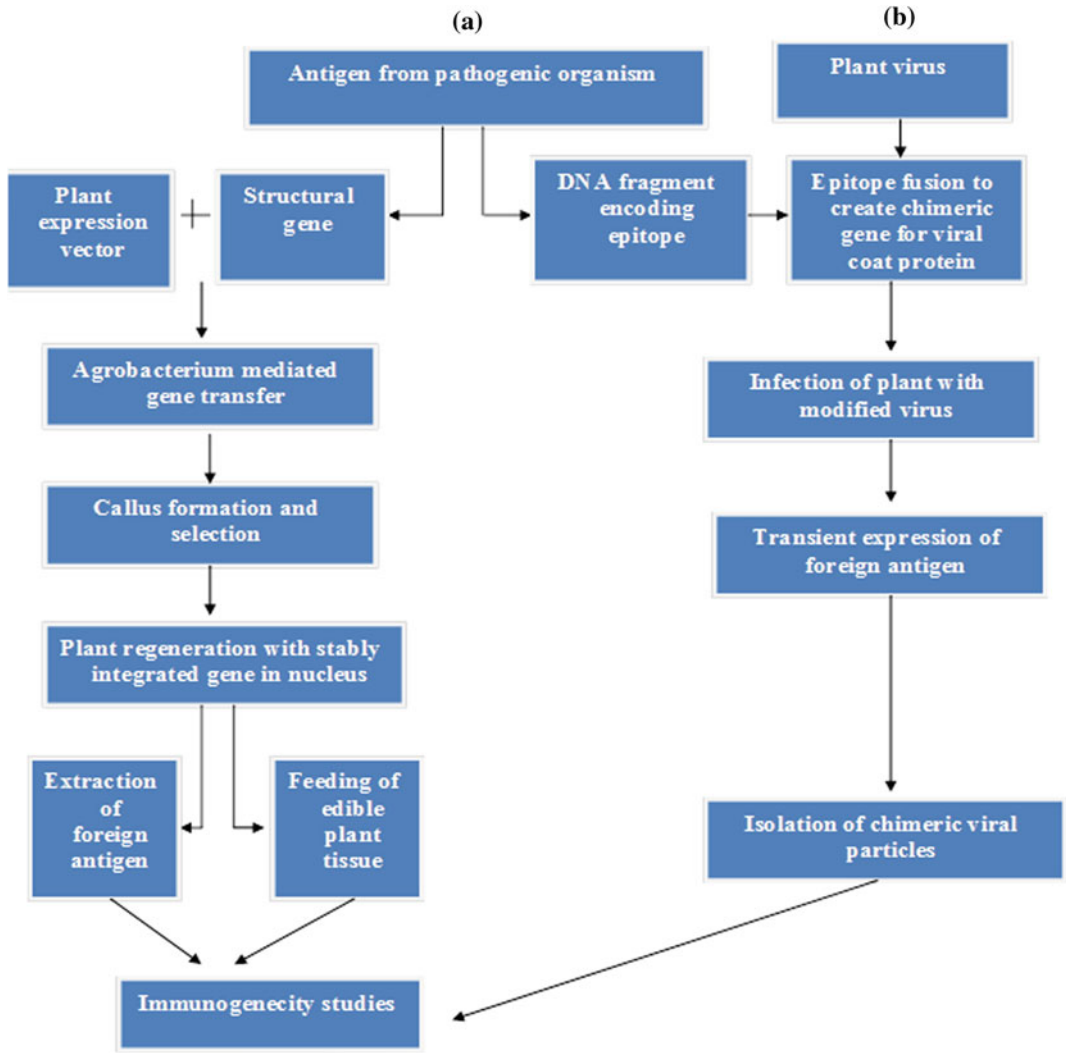


Fig. 12.1 (**a**) Stable and (**b**) transient strategies for the production of candidate vaccine in potato and tobacco plant tissue, respectively

engineering has also got impetus in last decade due to its various advantages over nuclear engineering.

2. Transient expression using viral vectors: In this method viral vectors are used as a tool to deliver genetic material into cells. A recombinant plant virus is selected that can carry the vaccine gene and can cause the plant to express the antigen by systemic infection. As compared to stable expression, transient expression is difficult to initiate, because the viral vectors must be inoculated into individual host plants, but gives higher level of expression as it allows the virus to replicate and amplify the gene copy number.

Some of the most popularly used techniques are described below:

12.7.1 *Agrobacterium tumefaciens* Mediated Gene Transfer Method

Plant transformation mediated by the plant pathogen, *A. tumefaciens* has become the most popular method lately. It is a naturally occurring

gram-negative soil bacterium, which infects the wound sites in dicot plants causing the formation of the crown gall tumor. This bacterium is capable of transferring a particular DNA segment (T-DNA) of the tumor-inducing (Ti) plasmid into the nucleus of infected cells where it is subsequently integrated into the host genome and transcribed. The T-DNA usually contains cancer-causing oncogenic genes and genes that synthesize opines which are excreted by infected crown gall cells and are a food source for bacterium. During the genetic manipulation, the Ti plasmid is engineered to carry the desired gene for vaccine and the virulent genes that cause tumor growth in plants are deleted. The transgene is integrated, expressed, and inherited in mendelian fashion. The whole plant can be then regenerated from individual transformed plant cell. It has been studied that genes are successfully expressed in experimental model plants and when given orally to animals, the extract of transgenic plant containing the antigen induced serum antibodies, thus can be used to produce the edible vaccine.

The application of *Agrobacterium* mediated transformation is at present possible to most species of agronomic interest, including members of family Graminae and Leguminosae. This opens interesting new aspect for the development of edible vaccines for both human and veterinary uses.

12.7.2 Biolistic Method

The second approach for nuclear transformation is based on the microprojectile bombardment method, also known as the gene gun or biolistic method. This method is especially beneficial for those plants which can not be transformed by *A. tumefaciens* mediated gene transfer method. Selected DNA sequences are precipitated onto metal microparticles and bombarded with a particle gun at an accelerated speed in a partial vacuum against the plant tissue placed within the acceleration path. Microparticles penetrate the walls and release the exogenous DNA inside the cell where it will be integrated in the nuclear genome. Thus, this method effectively introduces DNA. The cells that take up the desired DNA, are identified through the use of a marker gene (in plants the use of GUS is most common), and then cultured to replicate the gene and possibly cloned. This method has various advantages including (1) thousands of particles are accelerated at the same time causing multiple hits resulting in transferring of genes into many cells simultaneously, (2) since intact cells can be used, the difficulties encountered with the use of protoplast are automatically circumvented, and (3) the method is universal in its application so that cell type, size, and shape or the presence/absence of cell wall do not significantly alter its effectiveness.

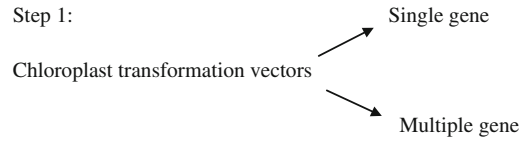
Another important use of the gene gun involves the transformation of organelles such as chloroplasts, and yeast mitochondria. The biolistic particle delivery system "shoots" adequately processed DNA particles, which penetrate into the chloroplast and integrate with its genome.

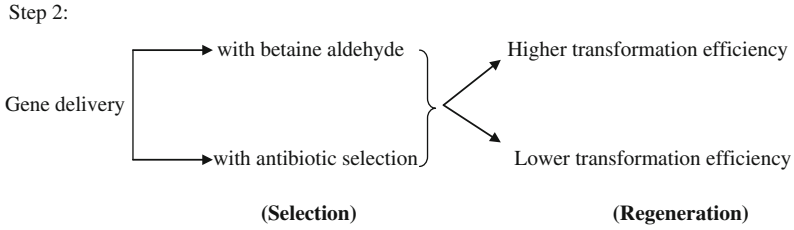
12.7.3 Chloroplast Transformation

The chloroplast's transformation is an interesting alternative to nuclear transformation which has come up in recent past. All plant cells have chloroplasts that capture light energy from the sun to produce free energy through a process called photosynthesis. In chloroplast genetic engineering, the recombinant DNA plasmid is bound to small gold nanoparticles that are injected into the chloroplasts of the leaf using a gene gun as described above. This device uses high pressure to insert the plasmid coated particles into the cells. These plasmids contain multiple genes of importance such as the therapeutic gene, a marker gene (may or may not be for antibiotic resistance), a gene that enhances the translation of therapeutic gene and two targeting sequences that flank the foreign gene. The foreign genes are inserted through homologous recombination *via* flanking sequences at a

precise and predetermined location in the organelle genome. The gene expression level in plastids is predominately determined by promoter and 5′-untranslated regions (5′-UTR elements) (Gruissem and Tonkyn 1993). Therefore, suitable 5′-UTRs including a ribosomal binding site (RBS) are important elements of plastid expression vectors (Eibl et al. 1999). In order to obtain high-level protein accumulation from expression of the transgene, the first requirement is a strong promoter to ensure high levels of mRNA. Most laboratories use the strong plastid rRNA operon (*rrn*) promoter (*Prrn*).

Besides gene gun, PEG mediated transformation and Galistan Expansion Femto Syringe microinjection techniques are also used for gene delivery in chloroplast. Some of the advantages of chloroplast transformation technology are its low cost, natural gene containment, site specific insertion, very high level of stable expression, generation of production lines with a competitive timeline, elimination of gene silencing, and high accumulation of the recombinant protein. Precise steps are given below:





Step 3:

A heteroplasmic diploid plant cell (First round of selection)

A homoplasmic diploid plant cell (Second round of selection)

Step 4:

Multiple gene expression

Step 5:

Reproductive organs

Disintegration of paternal plastids

Step 6:

Maternal inheritance of transgenic traits.

12.8 Mechanism of Action

The most common entry point for pathogens is at mucosal epithelia lining the gastrointestinal, respiratory, and urino-reproductive tracts, which are collectively the largest immunologically active tissue in body. The MIS is the first line of defense and the most effective site for vaccination against those pathogens; nasal, and oral vaccines being the most effective. The goal of oral vaccine is to stimulate both mucosal and humoral immunity against pathogens.

Edible vaccines have plant parts which are fed directly and the outer tough wall of plant cell acts to protect the antigens against attack by

enzymes, and gastric and intestinal secretions. This method is known as bioencapsulation. Therefore, the majority of the plant cell degradation occurs in the intestine as a result of action on digestive or bacterial enzymes. The antigens thus released are taken up by M cells in the intestinal lining that are present over the Peyer's patches (PP) in the ileum and the gut-associated lymphoid tissue. PP are an enriched source of IgA producing plasma cells and populate mucosal tissue and serves as mucosal immune effectors sites. The breakdown of edible vaccine occurs near PP, consisting of 30–40 lymphoid nodules on the outer surface of the intestine and contain follicles from which germinal center develops upon antigenic stimulation. These follicles act as the sites from which antigen penetrates the intestinal epithelium, thereby accumulating antigen within organized lymphoid structure. The antigens then come in contact with M cells which in turn express class II MHC molecules. Antigens transported across the mucous membrane by M cells can activate B-cells within these lymphoid follicles. The activated B-cells leave the lymphoid follicles and migrate to diffuse mucosal associated lymphoid tissue (MALT) where they differentiate into plasma cells that secrete the IgA class of antibodies. These IgA antibodies are transported across the epithelial cells into secretions of the lumen where they can interact with antigens present in the lumen and immediately neutralize the infectious agent. The induction of mucosal immunity by edible vaccine is depicted in the flow diagram (Fig. 12.2).

12.8.1 How Edible Vaccine Provides Protection?

An antigen in a food vaccine is taken up by M cells in the intestine and passed to various immune system cells, which then start a defensive attack, as antigen is a true infectious agent, not just part of one. The response leaves long-lasting "memory cells" able to promptly neutralize the real infectious agent. The whole procedure can be explained in two stages (Fig. 12.3 a, b) Antibodies and antibody fragments produced against specific antigens are given in Table 12.2.

12.9 Applications

There are numerous therapeutic and diagnostic applications of edible vaccines which are summarized in Table 12.3. Some of the diseases on which the work is going on are described below:

12.9.1 Malaria

Malaria is a disease of humans transmitted by the bite of an infected mosquito. It remains one of the most significant causes of human morbidity and mortality worldwide. According to WHO's 2010 world malaria report there are more than 225 million cases of malaria killing around 781,000 people. Three antigens are currently being investigated for the development of a plant-based malaria vaccine, merozoite surface protein (MSP) 4, MSP 5 from *Plasmodium falciparum* and MSP 4/5 from *P. yoelli.* Wang et al. (2004) have demonstrated that oral immunization of mice with recombinant MSP 4, MSP 4/5, and MSP 1, co-administered with CTB as a mucosal adjuvant, induces antibody responses effective against blood stage parasite.

12.9.2 Measles

Measles is an infection of the respiratory system caused by a virus. In an experiment mice fed with tobacco expressing MV-H (measles virus haemagglutinin from Edmonston strain) antibody titers five times the level considered protective for humans could be attained and secretory IgA was found in their feces. Prime boost strategy by combining parenteral and subsequent oral MV-H boosters could induce titers 20 times the human protective levels.

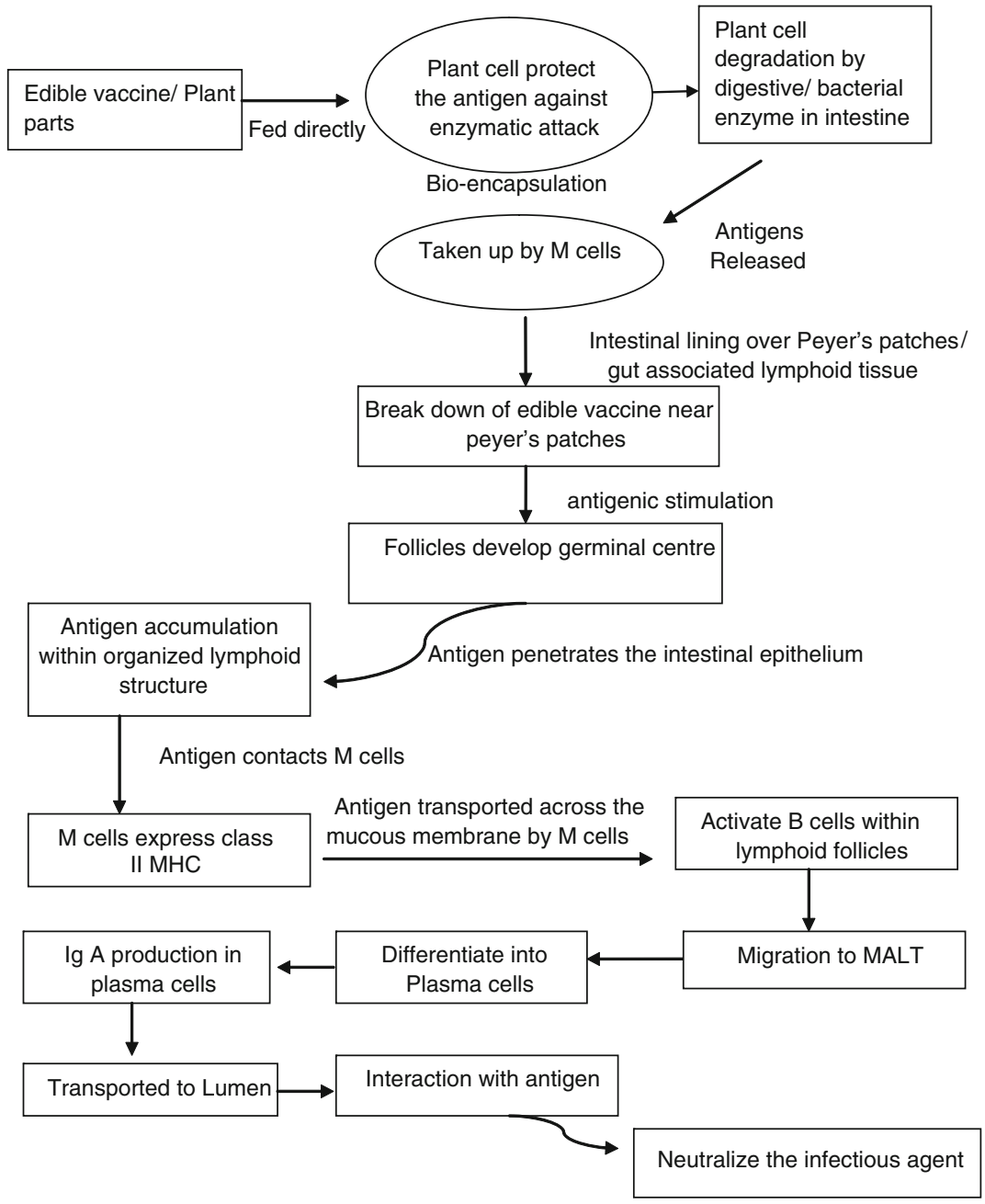


Fig. 12.2 Induction of mucosal immunity

These titers were significantly greater than with either of the vaccine administered alone. MV-H edible vaccine does not cause atypical measles, which may be occasionally seen with the current vaccine. Thus, it may prove better for achieving its eradication. The success in mice has prompted similar experiments in primates. Transgenic rice, and lettuce, and baby food against measles are also being developed. When given with CTB (adjuvant), 35–50 g MV-H lettuce is enough; however, an increased dose would be required if given alone.

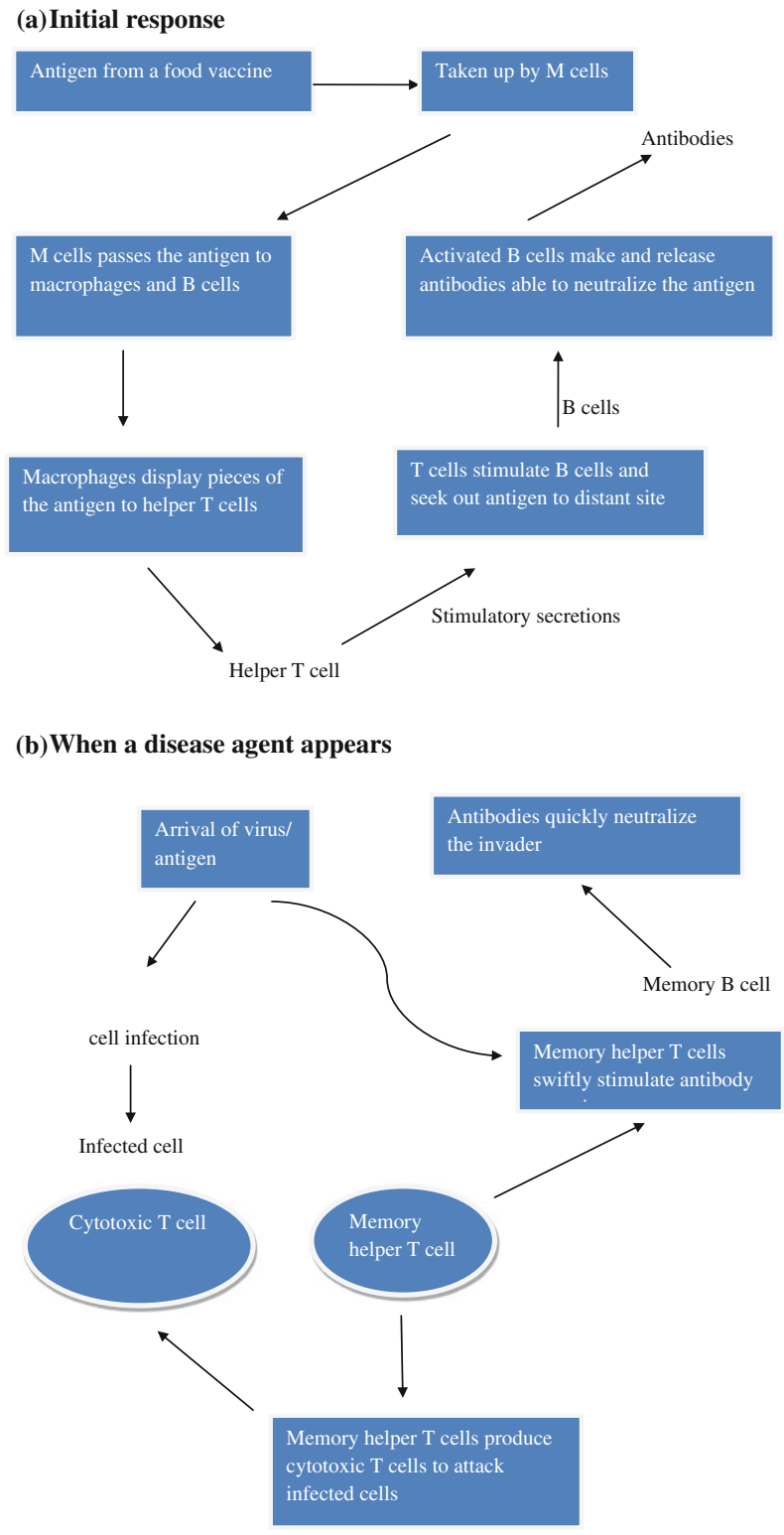


Fig. 12.3 **a** Production of antibodies. **b** Neutralization of invader

Table 12.2 Details of antigens produced by host plants and antibodies produced against them

Antibody	Antigen	Plant
IgG	Transition stage analog	Tobacco
IgM	NP(4-hydroxy-3-nitrophenyl) acetyl hapten	Tobacco
Single domain (dAb)	Substance B	Tobacco
Single chain Fv	Phytochrome	Tobacco
Single chain Fv	Artichoke mottled virus coat protein	Tobacco
Fab, IgG	Human creatine kinase	*Arabidopsis*
IgG	Fungal cutinase	Tobacco
IgG(k) and SIgG/A hybrid	*S. mutagens* adhesion	Tobacco
Single chain Fv	Abscisic acid	Tobacco
Single chain Fv	Nematode antigen	Tobacco
Single chain Fv	Alpha-glucuronidase	Tobacco
IgG	Glycoprotein B of herpes simplex virus	Soybean

(Das 2009)

Table 12.3 Therapeutic and diagnostic application of edible vaccines

Name of the vaccine	Vector	Pathological condition
Rabies virus	Tobacco, spinach	Rabies
Hepatitis B	Potato, Tobacco, Banana	Hepatitis B
HIV	Tomato	AIDS
Vibrio cholerae	Potato	Cholera
Cancer	Wheat, Rice	Cancer
Norwalk virus	Tobacco, potato	Hepatitis B
Rabbit hemorrhagic disease virus	Potato	Hemorrhage
Transmissible gastroenteritis corona virus	Tobacco	Gastroenteritis
Alzheimer's disease	Tomato	Alzheimer's disease
Colon cancer	Tobacco and potato	Colon cancer
Paramyxovirus	Banana, rice, lettuce	Measles
Plasmodium falciparum	Tobacco	Malaria
Type-I Diabetes	Potato	Type-I diabetes
Cysticercosis	Arabidopsis	Cysticercosis, foot and mouth disease

12.9.3 Rabies

Rabies is a deadly viral infection that is transmitted to humans from animals. Tomato plants expressing rabies antigens could induce antibodies in mice. Alternatively, TMV may also be used. Transformed tomato plants using CaMV with the glycoprotein (G-protein) gene of rabies virus (ERA strain) was shown to be immunogenic in animals.

12.9.4 Hepatitis B

Hepatitis B is a potentially life-threatening liver infection caused by the *hepatitis B* virus. It is estimated to have infected 400 million people throughout the globe, making the virus one of the most common human pathogen. First human trials of a potato-based vaccine against hepatitis B have reported encouraging results. Since immunization is the only known method to

prevent the disease of the hepatitis B virus, any attempt to reduce its infection requires the availability of large quantities of vaccine HBsAg. The amount of HBsAg needed for one dose could be achieved in a single potato. Levels of specific antibodies significantly exceeded the protective level of 10 mIU/mL in humans. When cloned into CaMV, the pCMV-S plasmid encoding the HBsAg subtype ayw showed higher expression in roots as compared to leaf tissue of the transgenic potato. Furthermore, expression of the antigen was found to be higher in roots of transgenic potato than in leaf tissues. However, the expression of HBsAg in transgenic potatoes is not sufficient for using as oral vaccine. Further studies are underway to increase the level of HBsAg by using different promoters e.g., patatin promoter, and different transcription regulating elements.

12.9.5 Cholera

Cholera is an infection of the small intestine that causes a large amount of watery diarrhea. It causes up to 10 million deaths per year in the developing world, primarily among children. Studies supported by WHO have demonstrated possibility of an effective vaccine for cholera, which provides cross protection against enterotoxic *E. coli*. To address this limitation, plants were transformed with the gene encoding B subunit of the *E. coli* heat liable enterotoxin (LT-B). Transgenic potatoes expressing LT-B were found to induce both serum and secretory antibodies when fed to mice; these antibodies were protective in bacterial toxin assay *in vitro*. This is the first "proof of concept" for the edible vaccine.

Since people eat only cooked potatoes, the effect of boiling on the properties of CTB expressed in transgenic potatoes was examined. After boiling for 5 min, over half of the vaccine protein survived in its biologically active form, providing evidence that cooking does not always inactivate edible vaccines. Thus, the spectrum of plants for producing edible vaccines may be expanded beyond raw food plants such as fruits. Co-expression of mutant cholera toxin subunit (mCT-A) and LT-B in crop seeds has been shown to be effective by nasal administration and is extremely practical.

12.9.6 Diabetes

The prevalence of *diabetes* is increasing globally and India is no exception. More than 100 million people are affected with diabetes worldwide. Type-I diabetes, also known as insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes, primarily affects children and young adults and accounts for 5–10 % of the diagnosed diabetes in North America. Research by Ma and Hein (1995) at the University of Western Ontario showed that diabetes can be prevented in mice by feeding them with plants engineered to produce a diabetes related-protein. The idea is based on 'oral tolerance' where the autoimmune system is selectively turned off early by teaching the body to tolerate the "antigenic proteins". The pancreatic protein, glutamic acid decarboxylase (GAD67) is linked to the onset of IDDM, and when injected into mice it is known to prevent diabetes.

The Canadian group developed transgenic potato and tobacco plants with the gene for GAD67, fed them to nonobese diabetic mice, which developed insulin-dependent diabetes spontaneously. The results were intriguing, only 20 % of the prediabetic mice fed with transgenic plants developed the diabetes, while 70 % non-treated mice developed the disease. The treated mice also showed increased levels of IG1, an antibody associated with cytokines, which suppresses harmful immune responses. Thus, the antigen produced in plants appears to retain immunogenicity and prevent diabetes in an animal model. According to Canadian scientists, this is the first proof of principle for the use of edible vaccines in the treatment of the autoimmune diseases.

12.9.7 HIV

Human immunodeficiency virus (HIV) is a retroviral that causes acquired immunodeficiency syndrome (AIDS), a condition in humans in which progressive failure of the immune system allows life-threatening opportunistic infections and cancer to thrive. In order to produce edible vaccine initial success in splicing HIV protein into CPMV has been achieved. Two HIV protein genes and CaMV as promoter were successfully injected into tomatoes with a needle, and the expressed protein was demonstrable by polymerase chain reaction (PCR) in different parts of the plant, including the ripe fruit, as well as in the second generation plants. Recently, spinach has been successfully inoculated for Tat protein expression cloned into TMV. Each gram of leaf tissue of spinach was shown to contain up to 300–500 μg of Tat antigen. Mice fed with this spinach followed by DNA vaccinations resulted in higher antibody titers than the controls, with the levels peaking at 4 weeks post-vaccination.

12.10 Regulatory Issues

It is still unclear whether the edible vaccines would be regulated under food, drugs. or agricultural products and what vaccine component would be licensed—antigen itself, genetically engineered fruit or transgenic seeds. They would be subjected to a very close scrutiny by the regulatory bodies in order to ensure that they never enter the food supply. This would include greenhouse segregation of medicinal plants from food crops to prevent outcrossing and would necessitate separate storage and processing facilities. Although edible vaccines fall under "Genetically modified" plants, it is hoped that these vaccines will avoid serious controversy, because they are intended to save lives.

12.10.1 Clinical Trials

Edible vaccines are future vaccines and some challenges are yet to be overcome before these can become a reality. Like all products regulated by Food and Drug Administration, edible vaccines undergo a rigorous review of laboratory, and clinical testing that are conducted to get information regarding safety, efficacy, purity, and potency of these products. These trials can take place only after satisfactory information has been collected on the quality of the nonclinical safety.

Successful expression of antigens in plants has been demonstrated in the past. The vaccines have also been checked for their efficacy in humans. Results from the primary phase of the first-ever human clinical trial of an edible vaccine were published in the journal Nature Medicine in 1998 (Blaine P. Friedlander, Boyce Thomson Institute of Plant Research), which indicated that consumption of servings of raw potatoes resulted in immunity to specific diseases. The human clinical study was conducted under the direction of Dr. Carol Tacket at the Center for Vaccine Development, University of Maryland School of Medicine in Baltimore. In the first phase of human testing, the potatoes eaten by volunteers contained a vaccine against travelers' diarrhea, a common condition resulting from intestinal infection by the bacterium *E. coli*, which contaminates food or water supplies. The clinical trials were approved in advance by the Food and Drug Administration.

Encouraged by the results of this study, scientists started exploring the use of this technique for administering other antigens. In 2005 Thanavala's group has developed a potato vaccine booster for use in conjunction with injected hepatitis B vaccine. It is currently in phase II clinical trial and phase I for patients who have previously been vaccinated. In 2000, Tacket and his team mates studied the human immune response to the Norwalk virus capsid protein expressed in potatoes. Overall, 95 % (19 out of 20 volunteers) developed some kind of immune response, although the antibody increase in some cases was modest. In same year, Pogrebnyak's lab developed an effective vaccine against the coronavirus which causes severe acute respiratory syndrome (SARS). Tomato and Tobacco plants are used for high expression of the coronavirus spike protein (S1). First, lyophilized tomato fruit was fed to mice and then boosting

occurred with S1 protein expressed in tobacco roots; high IgG1 immune responses and significant IgG2a and IgG2b responses were observed in their sera. Research is also going in the direction to engineer the plants to produce a variety of functional monoclonal antibody (Ma et al. 2005).

In the first human study of transgenic plant vaccine designed to induce active immunity, 14 adult volunteers were given either 100 g of transgenic potato, 50 g of transgenic potato or 50 g of wild type potato, each transgenic potatoes containing from 3.7 to 15.7 μg/g of LT-B. The variable dose per gram of potato was due to the tissue specificity of the promoter, therefore, that LT-B was expressed to a different degree in the different tissues of the potatoes. The potatoes in this study were ingested raw; however, subsequent studies have shown that transgenic potatoes expressing the B subunit of cholera toxin could be boiled for 3 min until the tissue becomes soft with loss of only about 50 % of the CT- B pentameric GM1-binding form. Serologic responses were also detected after vaccination. Totally 10 out of the 11 volunteers (91 %) who ingested transgenic potatoes developed IgG anti-LT and in half of them responses occurred after the first dose. There are 6 of the 11 (55 %) volunteers developed fourfold rise in serum IgA anti-LT.

Researchers supported by the NIAID have shown for the first time that an edible vaccine can safely trigger significant immune responses in people. The goal of the Phase 1 proof-of-concept trial study was to demonstrate that an edible vaccine could stimulate an immune response in humans. Volunteers ate bite-sized pieces of raw potato that had been genetically engineered to produce part of the toxin secreted by *E. coli,* which causes diarrhea. The trial enrolled 14 healthy adults, 11 were chosen at random to receive the genetically engineered potatoes and 3 received pieces of ordinary potatoes. The investigator periodically collected blood and stool samples from the volunteers to evaluate the vaccine's ability to stimulate both systemic and intestinal immune responses. The potatoes were well tolerated and no one experienced serious adverse side effects.

NIAID supported scientists are exploring the use of this technique for administering other antigens. Edible vaccines for other intestinal pathogens are already in the pipeline. Potatoes and bananas that might protect against Norwalk virus, a common cause of diarrhea, and potatoes and tomatoes that might protect against hepatitis B are being developed.

12.11 Future Perspective and Conclusion

Thirty million children throughout the world do not receive even the most basic immunizations each year. As a result, at least three million of these children die from diseases that are fully vaccine-preventable. The solution to vaccinate these children might seem simple with the idea of large scale production of edible vaccines for various diseases.

As a recent progress, the first human clinical trials for plant-based vaccine have been performed; it brings many challenges like optimization of expression levels, stabilization during post harvest storage, etc. Long-term reactions to edible vaccines are yet to be determined. Possible delayed reactions not yet discovered may be the point of consideration. In addition to that, edible vaccines can be further improved for their oral immunogenicity by the use of specific adjuvant which can be applied either as a fusion to the candidate gene or as an independent gene. Some of the diseases to which edible vaccines have shown promising application may be elaborated in the veterinary as well as human spectrum. These studies conclude plant-derived vaccines as a new hope and promise for more immunogenic, more effective, and less expensive vaccination strategies against both respiratory as well as intestinal mucosal pathogens.

Research in the field of edible vaccines holds immense potential for the future and every advancement made in this direction is bringing the dream of edible vaccine one step closer. There is hope that in coming future edible vaccines will conquer all serious diseases and make the planet beautiful to live in.

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