ISSN: 0975–833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 3, Issue, 5, pp.018-026, May, 2011

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

REVIEW ARTICLE

"EDIBLE" VACCINE-VEGETABLES AS ALTERNATIVE TO NEEDLES

[1]Ajaz Malik, [1]Vashisht, V.K., [1]Rizwan Rashid, [2]Susheel Sharma and [1]Jaskanwal Singh

[1]Department of Vegetable Crops, Punjab Agricultural University, Ludhiana 141004, India
[2]RARS (Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu) Rajouri, J&K

ARTICLE INFO

Article History:
Received 12th March, 2011
Received in revised form
19th April, 2011
Accepted 27th April, 2011
Published online 14th May 2011

Key Words:
Edible vaccines,
Needle-free,
Transgenic.

ABSTRACT

Edible vaccines or Food Vaccine hold great promise as an easy-to-administer, cost-effective, easy-to-store, and socio-culturally willingly acceptable vaccine delivery for the developing countries. Edible vaccines offer exciting opportunities for significantly reducing the burden of diseases like hepatitis, cholera & measles, mainly in the developing world where the administrating & storage of vaccines is at major concern. The search for methods of vaccine delivery not requiring a needle and syringe has been accelerated by recent concerns regarding pandemic disease, bioterrorism, and disease eradication campaigns. Needle□free vaccine delivery could aid in these mass vaccinations by increasing ease and speed of delivery, and by offering improved safety and compliance, decreasing costs, and reducing pain associated with vaccinations. Edible Vaccines are prepared by molecular farming with the help of genetic engineering. Preparation of Edible Vaccines are involving the introduction of selected desired genes into plants and inducing these genetically modified plants to manufacture the encoded proteins. This process is known as transformation and the altered plants are called transgenic plants**.** The vaccine is administered through the consumption of the edible plant as food, preferably in the form of a fruit or vegetable juice which can be taken orally.

INTRODUCTION

Immunization is a means of providing protective shield to the body, *i.e.* immunization or vaccination is a prophylactic approach through which the body is shielded or made strong enough to fight against any incoming pathogenic invasion. The process of distributing and administrating vaccines is referred to as vaccination. Vaccination is a form of immunization. Vaccines have been revolutionary for the prevention of infectious diseases. Despite worldwide immunization of children against the six devastating diseases, 20% of infants are still left un-immunized; responsible for approximately two million unnecessary deaths every year, especially in the remote and impoverished parts of the globe (Landridge, 2000). This is because of the constraints on vaccine production, distribution and delivery. One hundred percent coverage is desirable, because un-immunized populations in remote areas can spread infections and epidemics in the immunized "safe" areas, which have comparatively low herd immunity. For some infectious diseases, immunizations either do not exist or they are unreliable or very expensive. Immunization through DNA vaccines is an alternative but is an expensive approach, with disappointing immune response (Ramsay *et al.,* 1999). Hence the search is on for cost-effective, easy-to-administer, easy-to-store, fail-safe and socio-culturally readily acceptable vaccines and their delivery systems. As Hippocrates said, "Let thy food be thy medicine," There was a time not too long ago when most medicinal compounds came from plants.

***Corresponding author:** geemalik06@gmail.com*

But beginning about 50 years ago, chemistry took over from botany, with most new drugs being artificially produced in pharmaceutical labs (Moffat, 1995). Scientists suggest that plants and plant viruses can be genetically engineered to produce vaccines against diseases such as dental caries; and life-threatening infections like diarrhea, AIDS, etc (Moffat, 1995) . Nowadays, one of the most promising methods of producing proteins and other medicinal substances, such as antibodies and vaccines, is the use of transgenic plants. This is the concept of edible vaccines The search for methods of vaccine delivery not requiring a needle and syringe has been accelerated by recent concerns regarding pandemic disease, bioterrorism, and disease eradication campaigns. Needle□free vaccine delivery could aid in these mass vaccinations by increasing ease and speed of delivery, and by offering improved safety and compliance, decreasing costs, and reducing pain associated with vaccinations. Needle□free vaccination includes all methods for delivering vaccines that do not require a needle and syringe for administration. Transgenic plant "edible" vaccines offer promising vaccine delivery methods

Advantages and Disadvantages

Recombinant protein production using transgenic plants as bioreactors is likely to be more economical than alternative systems, especially for large-scale needs. Factors in favor of plant systems as sources of animal derived proteins, compared with other conventional methods, include:

a. Edible means of administration
b. Reduced need for medical persons and sterile injection conditions
c. Economic in mass production and transportation
d. Storage near the site of use
e. Heat stable eliminate the need for refrigeration
f. Generation of systemic and mucosal immunity
g. The potential for large-scale, low-cost biomass production using agriculture.
h. Low risk of product contamination by mammalian viruses, blood-borne pathogens, oncogenes and bacterial toxins.
i. The capacity of plant cells to correctly fold and assemble, not only antibody fragments and single chain peptides, but also full-length multimeric proteins.
j. Low downstream processing requirements for proteins administered orally.
k. Elimination of the purification requirement when the plant containing the recombinant proteins is edible, such as potatoes.
l. The ability to introduce new or multiple transgenes by sexual crossing of plants.
m. The avoidance of ethical problems associated with transgenic animals.
n. Formulated in seeds, plant-made enzymes have been found to be an extremely convenient method for reducing storage and shipping costs, for an indefinite amount of time, under ambient conditions.
o. Production size is flexible and easily adjustable to the needs of changing markets.
p. Plants are also capable of synthesizing and assembling virtually any kind of antibody molecule, ranging from the smallest antigen-binding domains and fragments, to full length, and even multimeric antibodies.

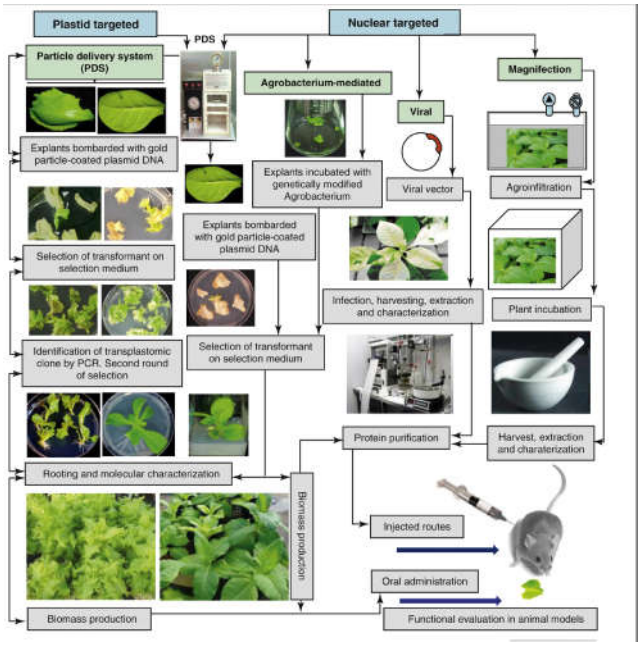
There are, however, potential issues of concern for plant protein production:

a. Allergic reactions to plant protein glycans and other plant antigens.
b. Plant and product contamination by mycotoxins, pesticides, herbicides and endogenous metabolites.
c. Regulatory uncertainty, particularly for proteins requiring approval for human drug use (Doran 1999).
d. Consistency of dosage from fruit , plant to plant, generation to generation is not similar
e. Stability of vaccine in fruit is not known
f. Evaluating dosage requirement is tedious.
g. Certain food like potato are not eaten raw, and cooking the food might weaken the medicine present in it.

MATERIALS AND METHODS

Conventional Method

Pharmaceutical and therapeutic antibodies synthesized in plants can be produced in a variety of ways. Conventional methods use stable transformation and transient expression to introduce new genes into a host cell. Once DNA from the transformant host cell is isolated and purified, it can be injected into the embryo of a maturing plant. The plant can then propagate in an open field allowing for large-scale production of antibodies. However, purification of these proteins is generally long and tedious. Upon isolation of the antibody, several proteins, organic molecules, glycan and herbicides must also be isolated, leading to a complex purification process (Kusnadi *et al.,* 1997).



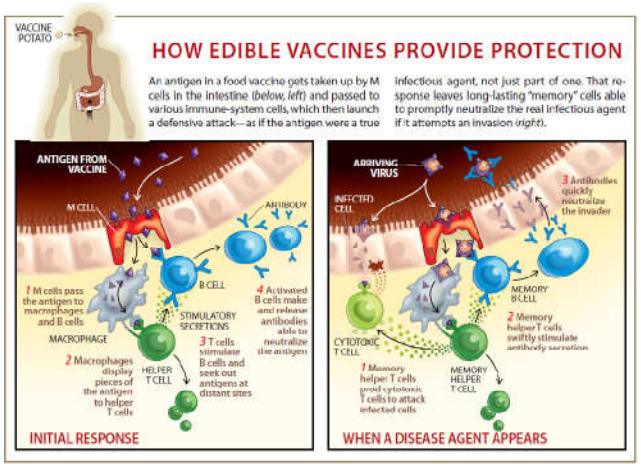
Creating edible vaccines involves introduction of selected desired genes into plants and then inducing these altered plants to manufacture the encoded proteins. This process is known as "transformation," and the altered plants are called "transgenic plants." Like conventional subunit vaccines, edible vaccines are composed of antigenic proteins and are devoid of pathogenic genes. Thus, they have no way of establishing infection, assuring its safety, especially in immune compromised patients. Introduction of foreign DNA into plant's genome can either be done by bombarding embryonic suspension cell cultures using gene-gun or more commonly through *Agrobacterium tumefaciens* , a naturally occurring soil bacterium, which has the ability to get into plants through some kind of wound (scratch, etc.). It possesses a circular "Ti plasmid" (tumor inducing), which enables it to infect plant cells, integrate into their genome and produce a hollow tumor (crown gall tumor), where it can live. This ability can be exploited to insert foreign DNA into plant genome. But prior to this, the plasmid needs to be disarmed by deleting the genes for auxin and cytokinin synthesis, so that it does not produce tumor. Genes for antibiotic resistance are used to select out the transformed cells and whole plants, which contain the foreign gene; and for expressing the desired product, which can then be regenerated from them. The DNA integrates randomly into plant genome, resulting in a different antigen expression level for each independent line, so that 50-100 plants are transformed together at a time, from which one can choose the plant expressing the highest levels of antigen and least number of adverse effects. Production of transgenic plants is species dependent and takes 3-9 months. Reducing this time to 6-8 weeks is currently under investigation. Some antigens, like viral capsid proteins, have to self-assemble into VLPs (virus-like particles). VLPs mimic the virus without carrying DNA or RNA and therefore are not infectious. Each single antigen expressed in plants must be tested for its proper assembly and can be verified by animal studies, Western blot; and quantified

by enzyme-linked immunosorbent assay (ELISA) (Haq *et al.,* 1995).

In Vitro Cell Tissue Cultures

Plant tissue cultures offer an economically favorable method for producing antibodies from plants. Using this approach, plant cells in differentiated or dedifferentiated states are grown in a nutrient medium in bioreactors under controlled conditions, with foreign proteins harvested from either the biomass or culture liquid or a combination of both (Doran, 1999). This method of production of human antibodies is not suitable for the production of edible vaccines (simply because the antibody is produced in a cell culture and not in a fruit or vegetable) but offers many advantages to the conventional methods of extracting and purifying a protein from a live plant. First, plant tissue cultures offer larger amounts of proteins in shorter amounts of time (Doran, 1999). This is because the bioreactors provide a much more controlled and reproducible environment than an open field. The advantages of this method allow desired antibodies to be produced, purified, and transferred to the consumer in a minimal amount of time. Also, purification of the proteins becomes easier (Doran, 1999). In vitro cell cultures contain fewer biological proteins or molecules (along with herbicides and pesticides) than open field plants or bacterial/yeast cell cultures, which may contaminate the product. Furthermore, plant cells and organs can propagate indefinitely in tissue cultures (Doran, 1999). Therefore, sexual reproduction is not needed to ensure the lifespan of the species. Without sexual reproduction, transgene stability is increased because of the absence of crossing over, segregation and recombination involved in sexual reproduction. Inducible promoters may offer a solution to the problems associated with open field production of plantibodies. They would allow for better efficiency in the production of plantibodies. They would also provide the plant with a more stable mode of translation, leading to transgene stability (Doran, 1999).

Mode of action of edible vaccines



A concern with oral vaccines is the degradation of protein components in the stomach (due to low pH and gastric enzymes) and gut before they can elicit immune responses (Daniell *et al.,* 2001) but the rigid plant cell walls could provide protection from intestinal degradation (Webster *et al.,* 2002). The degradation can be compensated by repeating the exposure of the antigen until immunological tolerance is accomplished (Mason *et al.,* 2002).The M cells lining the small intestine take in the components that have entered the small intestine (including pathogens) and pass them to other cells of the immune system, such as antigen presenting cells and macrophages. These cells chop up their acquisitions and display the resulting protein fragments on the cell surface. Helper T lymphocytes recognize the displayed fragments as foreign, induce B lymphocytes to secrete neutralizing antibodies and also help to initiate a broader attack on the perceived enemy. Mucosal immune responses represent a first line of defense against most pathogens. Edible vaccines activate both mucosal and systemic immunity, as they come in contact with the digestive tract lining. This dual effect would provide first-line defense against pathogens invading through mucosa, like *Mycobacterium tuberculosis* and agents causing diarrhea, pneumonia, STDs, HIV, etc.

Conventional vaccines Versus Vaccines made in plants

The functioning of vaccines made in plants is no different from that of conventional vaccines. Both produce antibodies to fight pathogens. So what ever be the vaccine that is injected in the body, this event is recorded by the immune system, so that at the later date if a specific pathogen invades the body the immune system produces a strong response to counter the pathogen. Conventional vaccines that are made from attenuated pathogens and involve the synthesis of antigenic proteins using mammalian cell culture which is easily prone to contamination with harmful pathogens. If microbial system is used to make vaccine there is possibility of endotoxin contamination. When cell culture and transgenic animals are used to make vaccines the contamination possibly arises with viruses, prions and oncogenic DNA. These processes involve the use of sophisticated and expensive sterile fermentation technology followed by purification processes. On the contrary, plant based systems only need green houses and not SS tanks for cell culture besides purification from plant extract are simpler because plants are not carriers of viruses that could possibly be detrimental to humans. As an example, a fermenter produced anthrax vaccine could get contaminated with Bacillus anthracis toxin. But if the same vaccine to be produced in plants it would be completely toxin free. In other words, a recipient of plant sourced vaccine is only exposed to non-infectious and non-toxic bit of protein .Although almost invariably it is the antigen that is recognized by the immune system, antigen alone cannot stimulate the immune system to elicit a sufficiently strong counter response but researcher have concluded that vaccines made in plant have an element of plant sugars that gives a markedly strong immune response. In that case the vaccine can get into the cell and stimulate the immune system to make more of WBCs. So vaccines sourced from plants give greater immune response because of the attachment of plant sugars antibodies. Although sugars also get attached to antibodies when vaccines are made from animal cells but that is not reckoned to be beneficial.

Vegetables as a candidate for edible vaccine

Tests showed that the transgenic vegetables created immune responses in both blood serum and the intestinal mucosa while choosing a plant for vaccine to be used as vaccine it is

important that it is a hardy, palatable plant with high nutritive and protein content the vegetable fits in this context to be used as edible vaccine. Potato was the first major system to be used for vaccine production, and transgenic potato tubers have been administrated into human clinical trials. During last few years potato have been evaluated for production of human serum albumin (Farran *et al.,* 2002), novel vaccine candidate (Yu *et al.,* 2003) and antibodies (Dewilde *et al.,* 2002). Tomato serves as ideal candidates for HIV antigen because unlike other transgenic plants that carry the protein, tomatoes are edible and immune, to ant thermal process, which helps retain its healing process. Lettuce is a fast growing species suitable for direct consumption and experimental studies, lettuce might replace booster shots in next generation.

and/or its edible portions has given a potential to explore further and expand the possibility of developing plants expressing more than one antigenic protein. Multi component vaccines can be obtained by crossing two plant lines harboring different antigens. Adjuvants may also be co-expressed along with the antigen in the same plant. B subunit of *Vibrio cholerae* toxin (VC-B) tends to associate with copies of itself, forming a doughnut-shaped five-member ring with a hole in the middle (Landridge, 2000). This feature can bring several different antigens to M cells at one time - for example, a trivalent edible vaccine against cholera, ETEC (Enterotoxigenic *E. coli*) and rotavirus could successfully elicit significant immune response to all three (Yu *et al., 2001*).

Table 1. Production of antigens in transgenic vegetables

Disease	Antigen	Transgenic plant	Remarks	Reference
Hepatitis B	HBsAg	Potato	Primary immune response was response was observed in mice	Richter *et al.*(2000)
Hepatitis E(HEV)	HEV-E2	Tomato	HEV-E2 gene was correctly expressed and antigen had normal immunoactivity	Ma *et al.* (2003)
Cholera	Cholera toxin B (CTB) sub unit fused to an ER retention signal	Potato	Immunological and biochemical properties were identical to native CTB protein	Arakawa et al (1997)
Viral enteric disease	CTB sub unit fused with rotavirus NSP4 protein	Potato	Synthisis and assembly of biologically active oligomers were observed	Kim and langridge (2003)
Murine rotavirus	Capsid structure protein VP6 of rotavirus	Potato	Oral immunization of mice induced detectable humoral and intestinal antibody	Yu and langridge (2003)
Measles	Loop –forming B cell epitope (H386-400) of the measles virus hemagglutinin protein	Carrot	Immunization of mice pritoneally with carrot plant extracts induce higher titers of antibodies	Bouche *et al.* (2003)
Cervical human papillomavirus-like particles (HPV) disease	HPV type 11 L1 major capsid protein	Potato	Oral immunization induced anti-VLP immune response in mice	Wazecha et al (2003)
Respiratory syncytial virus (RSV)	RSV-F protein	tomato	Oral immunization of mice induce both serum and mucosal RSV-F specific antibodies	Sandhu et al (2000)
Rabies virus	Rabies virus epitopes fused with tobacco mosaic virus	Spinach	Immunized mice showed immune response and protection from lethal dose of rabies virus	Modelska *et al.* (1998)
Anthrax	Lethal factor protein (LF), linked to CTB	Potato	CTB-LF assembled into biologically active pentamers	Kim *et al.* (2004)

Table 2. Advanta and Disvantages of Transgenic Vegetables

Vegetable	Advantages	Disadvantages
Potato	Dominated clinical trials easily manipulated/ transformed stored for long periods without refrigeration	Need cooking, which can denature antigens and decrease immunogenicity
Tomato	Grow quickly, cultivated broadly, high content of vitamin A ,heat- stable, antigen containing powders made into capsules Different batches blended to give uniform doses of antigen	Spoils readily
Lettuce	Fast-growing Direct consumption	Spoils readily
Musk melon (cantaloupe)	Fast growing easily transformed	Spoils readily

'Second-Generation' Edible Vaccines

Second generation edible vaccines are also called as multi component vaccines that provide protection against several pathogens Successful expression of foreign genes in plant cells

Global alliance for vaccines and immunization (GAVI) accords very high priority to such combination vaccines for developing countries.

Application of Edible vaccine

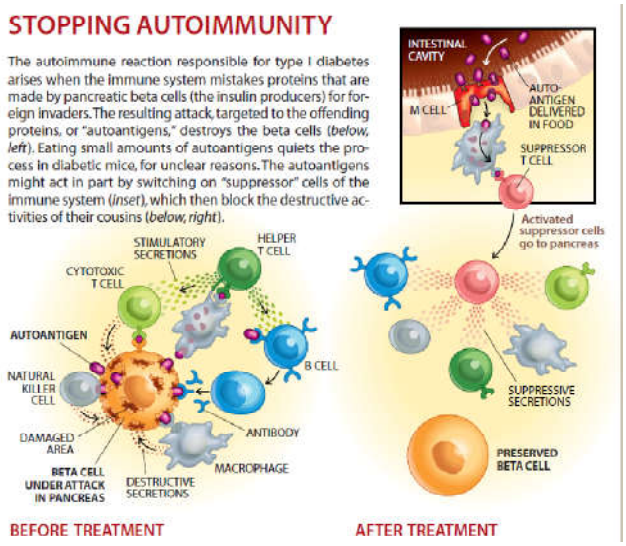
Malaria: Malaria, a disease caused by protozoan parasites of

genus Plasmodium, is one of the world's biggest scourges. Over two billion individuals reside in the malaria endemic areas and the disease affects 300–500 million people annually. As a result of malarial-infection, an estimated three million lives are lost annually, among them over one million children (majority under 5 years of age).The world malaria situation has become significantly worse in recent years as the main forms of malaria control, spraying programmes and chemotherapy, becoming less effective in the development of vector and parasite resistance. Three antigens are currently being investigated for the development of 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) has demonstrated that oral immunization of mice with recombinant MSP 4, MSP 4/5 and MSP1, co-administered with CTB as a mucosal adjuvant, induced antibody responses effective against blood-stage parasite. For those studies, however, protein was expressed in *E.coli* and protection was only evident when high dose antigen was administered. Whether oral delivery of a plant-derived malaria vaccine would induce significant immune responses in humans is uncertain. it has been suggested that antigen expression level in plants are so low that an unrealistic quantity of plant material would have to be consumed to achieve meaningful immunity. For this approach to bear fruits, transgenic technology has to improve antigenic expression to induce responses in susceptible population like children's with moderate food intake. Moreover, due to high levels of antigen anticipated to be necessary, it is likely that strong adjutants will also be required (Wang et al., 2004). Hence, appropriate adjuvants have to be identified and tested. Finally, in the face of reports showing induction of tolerance or immunity through comparable oral immunizations vaccination regimens must be rigorously tested in preclinical studies (Arakawa *et al.,* 1998).

Hepatitis B: The hepatitis B virus is estimated to have infected 400 million people throughout the globe, making it one of the most common human pathogens. 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, hepatitis B surface antigen (HbsAg). The HbsAg subtype ayw was cloned into CaMv plasmid and the regenerated plants from the transformed cells were shown to produce HbsAg. Furthermore, expression of the antigen was found to be higher in roots of the transgenic potato than in leaf tissues (Domansky, 1995). 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 the HbsAg by using different promoters such as the patatin promoter, and different transcription regulating elements

DIABETES

Diabetes is among the leading causes of death and shorten life expectancy by 20 years. More than 100 million people are effected with diabetes world wide. Type 1 diabetes also known as insulin dependent diabetes mellitus (IDDM), primarily effect children's and young adults .it is an autoimmune disease where the pancreatic beta cells which produce insulin are destroyed by the body's own immune system. Research by Ma et al., 1995 at the university of western Ontario showed that diabetes can be prevented in mice by feeding then with plants engineered to produce a diabetes-related protein. The idea is based on oral tolerance where the auto immune system is selectively turned off early by teaching the body to tolerate the antigenic proteins. The pancreatic protein, gluatmic acid decarboxylase (GAD67), is linked to the onset of IDDM, and when injected into mice it is known to prevent diabetes. The scientists have developed transgenic potato with the gene for GAD67, feed them to non-obese diabetic mice, which develop insulin-dependent diabetes spontaneously. The result were intriguing: only 20% of the pre diabetic mice feed with transgenic plants develop the diabetes while 70% non treated mice developed the disease. The treated mice also showed increased level of IGI, an antibody associated with cytokines, which suppresses harmful immune responses. Thus the antigen produced in plant appears to retain immunogenicity and prevent diabetes in an animal model.



Cholera

Cholera and other diarrhoeal diseases caused upto ten million deaths per year in the developing world, primarily among children. Relatively little work on vaccines, among children to prevent these diseases is underway, study supported by WHO has demonstrated possibility of an effective vaccine for cholera, which provide 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 potato 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* .Since people eat only cooked potatoes, the effect of boiling on the properties of CTB expressed in transgenic potatoes was examined. After boiling for five minutes, 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 the plant for producing edible vaccines may be expanded beyond raw food plants such as fruits (Mason *et al.,* 1998)

Chemical Trials

Antigen expression in plants has been successfully shown in the past, like LT-B (ETEC) in potato, rabies virus-G protein in tomato (Tripurani *et al.,* 2003), HBsAg in tobacco (*Mason*

et al., 1992) and potato, norwalk virus in tobacco and potato; CT-B (*Vibrio cholerae*) in potato (Landridge, 2000). Ethical considerations usually preclude clinical trials from directly assessing protection, except in a few cases (Mason *et al.,* 1998). In contrast, veterinary researchers can assess immune protection more directly.

ETEC

Charles Arntzen at Boyce Thompson Institute, USA, accomplished the first published successful human trial in 1997 (Tacket *et al.,* 1998). Eleven volunteers were fed raw transgenic potatoes expressing LT-B. Ten (91%) of these individuals developed neutralizing antibodies and six (55%) developed a mucosal response.

Norwalk virus

Nineteen (95%) out of 20 people fed with transgenic potato expressing norwalk virus antigen showed seroconversion (Tacket *et al.,* 1998). Attempts are underway to engineer bananas and powdered tomatoes expressing norwalk virus.

Cholera

Transgenic potato with CT-B gene of *Vibrio cholerae* was shown to be efficacious in mice. Eating one potato a week for a month with periodic boosters was said to provide immunity. Co-expression of mutant cholera toxin subunit A (mCT-A) and LT-B in crop seed has been shown to be effective by nasal administration and is extremely practical (Yuki and Kiyono, 2003).

Measles

Mice fed with tobacco expressing MV-H (measles virus haemagglutinin from Edmonston strain) could attain antibody titers five times the level considered protective for humans and they also demonstrated secretory IgA in their faeces (Huang, 2001) .Prime boost strategy by combining parenteral and subsequent oral MV-H boosters could induce titers 20 times the human protective levels. These titers were significantly greater than with either vaccine administered alone (Webster et al., 2002). MV-H edible vaccine does not cause atypical measles, which may be occasionally seen with the current vaccine (Polack, 1999). Thus it may prove better for achieving its eradication. The success in mice has prompted similar experiments in primates.

Transgenic rice, lettuce and baby food against measles are also being developed. When given with CT-B (adjuvant), 35-50 gm MV-H lettuce is enough; however, an increased dose would be required if given alone (Giddings, 2000).

Hepatitis B

For hepatitis B, parenteral VLPs could invoke specific antibodies in mice (Thanavala *et al.,* 1995). First human trials of a potato-based vaccine against hepatitis B have reported encouraging results. 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 (cauliflower mosaic virus), plasmid HBsAg subtype ayw showed higher expression in roots as compared to leaf tissue of the transgenic potato. Further studies are required to increase the production of antigen by using different promoters, like patatin promoter. The resulting plant material proved superior to the yeast-derived antigen in both priming and boosting immunity in mice. Prime boost strategy in mice with a single sub-immunogenic parenteral dose of yeast-derived recombinant HBsAg and subsequent oral transgenic potatoes led to the development of antibodies that immediately peaked at >1,000 mIU/mL and were maintained at >200 mIU/mL for five months. This could be a useful immunization strategy for developing countries (Richter *et al.,* 2000). Tomatoes expressing hepatitis B are being grown in guarded greenhouses. Enough antigens for 4,000 vaccine doses were obtained from just 30 tomato plants. Transgenic lettuce is also being developed.

Rabies

Tomato plants expressing rabies antigens could induce antibodies in mice (Prakash *et al.,* 1996). 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.

HIV

Initial success in splicing HIV protein into CPMV has been achieved (Prakash, 1996). 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-

Table 3. Patents on edible vaccine technologies

S.No	Patent holder	Claim	Reference
1	Prodigene	Vaccine produced in genetically engineered plants for hepatitis and transmissible gastroentertits virus	www.prodigene.com
2	Scripps Research Institute	Recombinant antigen production in lettuce, spinach, kidneybean	www.prodigene.com
3	USDA/Univ.Philadelphia	Rabies vaccine expressed in tomato plant	www.prodigene.com
4	University of Loma linda	Gene constructs used to produce edible vaccines to treat autoimmune diseases	www.prodigene.com
5	Ribozyme-Pharm	Nucleic acid vaccine used to treat viral infection in plants, animals or bacteria	www.prodigene.com
6	Rubicon -Lab	Retrovirus expressed in animal or plant cells useful as virus and cancer vaccine	Toone, (1996)
7	University of Yales	Vaccine against invertebrates	Khoudi *et al.,* (1999)
8	University of Texas	Hepatitis B virus core antige recombinant vaccine	Pollacck, (2000)
9	Biocem	Rabies vaccine in transgenic plant	www.prodigene.com
10-	Cornell university	Increasing foreign protein expression	Khoudi *et al.,* (1999)

generation plant. 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 mg of Tat antigen (Karasev *et al.,* 2005). Mice fed with this spinach followed by DNA vaccinations resulted in higher antibody titers than the controls, with the levels peaking at four weeks post-vaccination.

STDs

Human papilloma virus type-11 (HPV-11) recombinant VLPs produced in insect cells are immunogenic when given orally to BALB/c mice. The response is dose-dependent, conformationally-dependent and genotype-restricted. Thus, VLPs may be effective oral immunogens for the prevention of anogenital HPV disease.

Anthrax

Tobacco leaves bombarded with pag gene (anthrax protective antigen - PA) using a gene gun could express a protein structurally identical to the major protein present in existing vaccine. Billions of units of anthrax antigen could be produced. In addition, this vaccine was devoid of edema factor and lethal factor, responsible for the toxic side effects. The same anthrax antigen is now being put in tomato plants. Scientists are also trying to transform spinach by inoculating it with TMV-expressing PA, as spinach might be a safer vaccine.

Challenges

The challenges facing plant-based-vaccine development include technical, regulatory and economic aspects and public perception. Among the technical challenges it is critical to select a plant system that can be grown under conditions that minimize environmental risks, such as transfer of pollen from transgenic to conventional varieties or to related species. Expression of antigens in plants is a major regulatory concern. Whether or not the protein is confined to specific tissues will enable or nullify exposure to the environment. Targeting expression via a tissue-specific promoter driving the transgene may reduce regulatory concerns (Korban, 2002). For example, elimination of expression of the transgene in pollen will reduce dissemination of the antigenic protein to other plants and alleviate environmental contamination, although not completely. Among other technical challenges to be considered, the crop should provide ample biomass for accumulation of a sufficient quantity of the antigenic protein. Whether it is a grain, vegetable or fruit crop, protocols will be needed to ensure transcription, translation, intracellular localization, tissue specificity, adequate gene-copy number, and metabolism and accumulation of the protein of interest (Streatfield and Howard, 2003). Among regulatory challenges, issues relevant to any genetically modified (GM) crop that have to gain regulatory approval from the USDA, FDA and/or EPA apply to plantbased vaccines. In addition, issues related to separation of a pharmaceutical product from the original crop targeted for the food chain have become increasingly important as concerns over adventitious presence of medicinal products in the food supply have surfaced in recent years. Physical separation of dual-purpose crops is needed—whether achieved by geographical isolation or by greenhouse containment—as is dedicated equipment for harvesting and handling, as well as standardized monitoring procedures.

Table 4. Data and Information Typically Required For Regulatory Review of the Potential Safety in Food of a Plant GMO

Feature of the GMO	Example Data Sets and Information
Parent organism	History of safe use as a food
Donor organism	Known pathogenicity and previous history of safe use as a food, potential allergenic properties
Altered phenotype	The encoded protein and its function
Introduced genetic material	Description of the vector, description of genetic elements, sequence of the vector, the transformation process, sequence of flanking regions at insertion
Altered phenotype	Description, morphology compared to parental organism, any intended levels of nutrient alteration, variation in concentration of nutrients in different environments, potential for modification of the modified phenotype (for example after processing)
Genetic modification	Southern analysis, Northern analysis, measure of expression of the transgene such as Westerns, immunoblotting
Detection technique	PCR, Biochemical assay
Dietary intake	How the food will be processed and consumed
Nutritional data	Key metabolite concentrations, comparison to nutrient levels in other foods
Toxicological data	Sequence comparison to databases, similarity to know toxins, levels of exposure, heat stability of introduced protein, simulated digestion studies, acute oral toxicity studies, animal feeding studies
Allergenicity	Results of immune tests, similarity of protein to know allergens, resistance of protein to heat and digestion
Horizontal gene transfer	Assessment of interaction with gut microflora

Table 5. Internet Access Points to Legislation of GMO in Key Countries

Country	Agency responsible for environmental safety	Agency responsible for food safety	Portal details
United States	Department of Agriculture (APHIS), Environmental protection agency (EPA)	The Food and Drug Administration (FDA)	www.aphis.usda.gov/brs
Canada	Canadian Food Inspection Agency	Canadian Food Inspection Agency	www.inspection.gc.ca
Australia	Office of the Green Technology regulator (OGTR)	Food Standards Australia and New Zealand (FSANZ)	www.foodstandards.gov.au
European Union	Several Community-wide directives	Several Community-wide directives	Biotech.jrc.it;
Japan	Ministry of Education, Culture, Sports, Science and Technology (NEXT)	Ministry of Agriculture, Forestry and Fisheries of Japan	www.maff.go.jp
China	Ministry of Agriculture, National Environment Protection Agency	Ministry of Public Health	www.agri.gov.cn
India	Ministry of Science and Technology, Ministry of Environment and Forestry	Ministry of Science and Technology	dbtindia.nic.in
Russia	Ministry of Science and Technology	Ministry of Agriculture and Food	www.aris.ru
Argentina	Agricultural Directorate of the Secretariat of Agriculture, Livestock, Fisheries, and Food	Agricultural Directorate of the Secretariat of Agriculture, Livestock, Fisheries, and Food	www.sagpya.mecon.gov.ar

Conclusion

Producing vaccines in plants offers numerous advantages over current vaccine methodologies.Among them, safety, ease of production and low cost of production provide strong justification for developing the technology. However, many challenges remain within the pharmaceutical industry; requirements for generating non-food products in transgenic plants are different from those for food products. These challenges include technical, regulatory, economic, and public-perception issues. Physical isolation, delayed planting, agronomic support, dedicated equipment and frequent monitoring all contribute to the technical challenges involved. As the technology to produce vaccines in plants goes through the regulatory pathway and demonstrates its economic feasibility, it may also overcome public-perception concerns that seem to have been dodged by the pharmaceutical industry. The likelihood that plant-based vaccines can be administered

via oral or intranasal delivery systems will also add to their desirability as well as their economic benefits. There is potential for major impacts on global health, particularly in developing countries. However, standardized safety-assessment models must meet with approval from the general public along with the regulatory agencies and other interested parties. Risk assessment must be science-based in order for the results to be believable and trustworthy. Funding of research will accelerate the advances made thus far, and bring this technology closer to commercialization and worldwide use.

REFERENCE

Arakawa, T., Chong, D.K.X., Merritt, J.L., Langridge, W.H.R. 1997. Expression of cholera toxin-B subunit oligomers in transgenic potato plants. *Transgenic Res.,* 6 : 403-413.

Arakawa, T., Yu, J., Chong, D.K., Hough, J., Engen, P.C. et al. 1998. A plant-based cholera toxin B subunit-insulin fusion protein protects against the development of autoimmune diabetes. *Nat. Biotechnol.,* 16 : 934-938.

Blanas, E., Carbone, F.R., Allison, J., Miller, J.F. and Heath, W.R. 1996. Induction of autoimmune diabetes by oral administration of auto antigen. *Science,* 274 : 1707-1709.

Bouche, F.B., Marquet-Blouin, E., Yanagi, Y., Steinmetz, A., Muller, C.P. 2003. Neutralising immunogenicity of a polyepitope antigen expressed in a transgenic food plant: a novel antigen to protect against measles. *Vaccine,* 21: 2074-2081.

Daniell, H., Streatfield, J., Wycoff, K. 2001. Medical Molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. *Trends in Plant Sci.,* 6: 219-226

Dewilde, C., Peeters, K., Jacobs, A., Peck, L. and Depicker, A. 2002. Expression of antibodies and Fab fragments in transgenic potato plants: A case study for bulk production in crop plants. *Mol. Breed*., 9 : 271-282.

Domansky, N. 1995. Organ-Specific Expression of Hepatitis B Surface Antigen in Potato. *Biotechnol. Lett.,* 17:863-866.

Doran, P.M. 1999. Foreign protein production in plant tissue cultures; *Current Opinion in Biotechnology,* 11: 199-204.

Farran, L., Sanchez-Serrano, J.J., Medina, J.F., Prieto, J. & Mingo-Castel, A.M. 2002. Targeted expression of human serum albumin to potato tubers.*Transgenic Res.,* 11: 337-346.

Giddings, G., Allison, G., Brooks, D., Carter, A. 2000. Transgenic *plants as factories for biopharmaceuticals.* Nat. Biotechnol., 18 :1151-1155.

Haq, T.A., Mason, H.S., Clement, J.D. et. al. 1995. Oral Immunization with a Recombinant Bacterial Antigen Produced in Transgenic Plants; *Science,* 268: 714-716.

Hassler, S. 1995. Bananas and Biotech Consumers. *Bio/Technology,* 13: 417.

Huang, Z., Dry, I., Webster, D., Strugnell, R., Wesselingh, S. 2001. Plant-derived measles virus hemagglutinin protein induces neutralizing antibodies in mice. Vaccine, **19** :2163-2171.

Karasev, A.V., Foulke, S., Wellens, C., Rich, A., Shon, K.J., Zwierzynski, I., et al. 2005. Plant based HIV-1 vaccine candidate: *Tat protein produced in spinach*. 23 :1875-1880.

Khoudi, H., Laberge, S., Ferullo, J., Bazin, R., Darveau, A. et al. 1999. Production of a diagnostic monoclonal antibody in perennial alfalfa plants. *Biotechnol Bioeng.,* 64 : 135-143.

Kim, T.G., Galloway, D.R., Langridg, W.H. 2004. Synthesis and assembly of anthrax lethal factor-cholera toxin B-subunit fusion protein in transgenic potato. *Mol. Biotech.,* 28 : 175-183.

Kim, T.G., Langridge, W.H. 2003. Assembly of cholera toxin B subunit full-length rotavirus NSP4 fusion protein oligomers in transgenic potato. *Plant Cell Rep.,* 21 : 884-890.

Korban, S.S. 2002. Targeting and expression of antigenic proteins in transgenic plants for production of edible oral vaccines. *In Vitro Cellular and Developmental Biology–Plant,* 38: 231–236.

Kusnadi, A.R., Nikolov, Z.L., Howard, J.A. 1997 Production of recombinant proteins in transgenic plants: practical considerations. *Biotechnology and Bioengineering*, 56: 473-84

Landridge, W. 2000. Edible vaccines. Scientific Am., 283 : 66-71.

Ma, J.K.C., Hiatt, A., Hein, M., Vine, N.D., Wang, F. et al. 1995. Generation and assembly of secretory antibodies in plants. *Sciences*, 268 : 716-719.

Ma, Y., Lin, S.Q., Gao, Y., Li, M., Luo, W.X., Zhang, J., Xia, N.S. 2003. Expression of ORF2 partial gene of hepatitis E virus in tomatoes and immunoactivity of expression products. *World J. Gastroenterol.,* 9 : 2211-2215.

Mason, H.S., Haq, F.A., Clement, J.D. and Amtzen, C.J. 1998. Edible vaccine protects mice against *Escherichia coliheat*-labile exterotoxin (LT) : Potatoes expressing a synhthetic LT-B gene. *Vaccine* 16(1998) : 1336-1343.

Mason, H.S., Lam, D.M., Arntzen, C.J. 1992. Expression of hepatitis B surface antigen in transgenic plants. Proc. Natl. Acad. Sci. USA, 89 :11745-11749.

Mason, H.S., Warzecha, H., Tsafir, M.S., Arntzen, C.J. 2002. Edible plant vaccines: applications for prophylactic and therapeutic molecular medicine. *Trends Mol. Med.,* 8:324-329.

Modelska, A., Dietzschold, B., Sleysh, N., Fu, Z.F., Steplewski, K., Hooper, D.C., Koprowski, H. Yusibov, V. 1998. Immunization against rabies with plant-derived antigen. *Proc. Natl. Acad. Sci.* USA 95 : 2481-2485.

Moffat, A.S. 1995. Exploring transgenic plants as a new vaccine source. Science, 268: 658-60.

Polack, F.P., Auwaerter, P.G., Lee, S.H., Nousari, H.C., Valsamakis, A., Leiferman, K.M., *et al.* 1999. Production of atypical measles in rhesus macaques: Evidence for disease mediated by immune complex formation and eosinophils in the presence of fusion-inhibiting antibody. *Nat. Med.*,5 : 629-634.

Pollack, A. 2000. Ventures aim to put farms in pharmaceutical vanguard. The New York Times, May 14.

Prakash, C.S. 1996. Edible vaccines and antibody producing plants. *Biotechnol. Develop. Monitor,* 27 :10-3.

Ramsay, A.J., Kent, S.J., Strugnell, R.A., Suhrbier, A., Thomson, S.A., Ramshaw, I.A. 1999. Genetic vaccination strategies for enhanced cellular, humoral and mucosal immunity. *Immunol. Rev.,* **171:** 27-44.

Richter, L.J., Thanavala, Y., Arntzen, C.J. 2000. Mason HS. Production of hepatitis B surface antigen in transgenic plants for oral immunization. *Nat, Biotechnol.,* 18 :1167-71.

Richter, L.J., Thanavala, Y., Arntzen, C.J., Mason, H.S., 2000. Production of hepatitis B surface antigen in transgenic plants for oral immunization. *Nat. Biotech.,* 18: 1167-1171.

Sandhu, J.S., Krasnyanski, S.F., Domier, L.L., Korban, S.S., Osadjan, M.D., Buetow, D.E. 2000. Oral immunization of mice with transgenic tomato fruit expressing respiratory syncytial virus-F protein induces a systemic immune response. *Transgenic Res.,* 9: 127-135.

Streatfield, J., Howard, J.A. 2003. Plant production systems for vaccines. *Expert Review of Vaccines*, 2: 763–775.

Tacket, C.O., Mason, H.S., Losonsky, G., Clements, J.D., Levine, M.M., Arntzen, C.J. 1998. Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. *Nat. Med.* 4 : 607-609.

Tacket, C.O., Mason, H.S., Losonsky, G., Estes, M.K., Levine, M.M., Arntzen, C.J. 2000. Human immune responses to a novel Norwalk virus vaccine delivered in transgenic potatoes. *J. Infect. Dis.,* 182 :302-305.

Thanavala, Y., Yang, Y.F., Lyons, P., Mason, H.S., Arntzen, C.J. 1995. Immunogenicity of transgenic plant-derived hepatitis B surface antigen. Proc. Natl. Acad. Sci. USA, 92:3358-61.

Toonen, J. 1996. Seeds of a new medicine. Biotechnology and Development Monitor No. 27 : 12-14.

Tripurani, S.K., Reddy, N.S., Sambasiva Rao, K.R. Green revolution vaccines, edible vaccines. *Afr J Biotechnol.,* 2003; **2:** 679-83.

Wang, L., Goschnick, M.W. & Coppel, R.L. 2004. Oral immunization with a combination of *Plasmodium yoelii* merozoite surface proteins 1 and 4/5 enhances protection against lethal malaria challenge. Infect Immunol, 72 : 6172-6175.

Warzecha, H., Mason, H.S., Lane, C., Tryggvesson, A., Rybicki, E., William-son, A.L., Clements, J.D., Rose, R.C.J. 2003. Oral immunogenicity of human papillomavirus-like parties expressed in potato. *Virology*, 77 : 8702-8711.

Webster, D.E., Thomas, M.C., Strugnell, R.A., Dry, I.B., Wesselingh, S.L. 2002. Appetising solutions: an edible vaccine for measles. *Med. J. Aust*., 176: 434-437.

Yu, J. and Langridge, W. 2003. Expression of rotavirus capsid protein VP6 in trangenic potato and its orgal immunogenicity in mice. *Transgenic Res.,* 12 : 163-169.

Yu, J., Langridge, W.H. 200.1 A plant-based multicomponent vaccine protects mice from enteric diseases. *Nat. Biotechnol.,* 19:548-552.

Yu, J., Langridge, W.H., 20001.A plant-based multicomponent vaccine protects mice from enteric diseases. *Nat. Biotechn.,* 19: 548-552.

Yuki, Y., Kiyono, H. 2003. New generation of mucosal adjuvants for the induction of protective immunity. *Rev.Med Virol*., 13:293-310.

****