Chapter 5

Production of Recombinant Proteins from Plants - An Overview

Mathangi Ganapathy*; Perumal Kaliraj

**Centre for Biotechnology, Anna University, Chennai, 600025.*

Email: mathangig@gmail.com

1. Introduction

Since time immemorial, mankind has been developing crops to suit their needs by selective breeding. Cultivar development within a species has been done by selecting alleles present a population. Sexual recombination allows alleleic recombination during meiosis resulting in plants which can be selected with novel traits. The primary gene pool allows for crosses to be easily made, the hybrids will be fertile and chromosome pairing is normal, thus allowing Mendelian segregation of traits. The biological species is divided into two categories, (i) subspecies composed of lines used for agronomic use and (ii) subspecies, which contains weedy or wild relatives of subspecies A. The secondary gene pool refers to all biological species, which cross with a different species. Although mating and gene transfer are possible, there will be hybrid sterility or aberrations in chromosome pairing. Hybrids may be weak and may not reach maturity. The recovery of a desired phenotype will not be possible or might be difficult even when traits are crossed back. In the tertiary gene pool, although crosses are possible with the crop of interest, hybrids are lethal or completely sterile. Hybrids can be rescued by embryo rescue, grafting, the application of mutagens to break apart chromosomes or by doubling the chromosome number. The quaternary gene pool does not allow any transfer of DNA between the crop of interest or other organism by mating and sexual recombination. In this case, genetic transformation would be the only way to insert a DNA sequence from any biological or synthetic source into the crop plant of interest. The DNA can be chimeric where different segments e.g. the promoter, gene of interest and terminator can be taken from any source and put together in a functional manner so that the DNA cassette is expressed once transferred into the genome of the crop of interest.

Genetic engineering of plants have been done from the early 80s. This has become possible because of the emergence of new technologies which enables the incorporation of genes

into plant cells or organelles for transient as well as stable expression and exploiting the totipotency of the plant cell. The traits introduced into plants can be for increasing the agronomic traits like improved nutritional quality, altered metabolic pathways for nutraceutical production, reduce the maturation time of crops or altering the protein profile in food crops or develop plants as expression platforms for the production of recombinant therapeutic proteins. Plants produce large biomass hence plants can produce large quantities of recombinant proteins at low cost, this would be commercially viable. At the same time, care has to be taken about the contamination of food crops or products because of transgene integration and expression. Humans may develop immunotolerance due to oral dosage of vaccine as well , also illegal or unethical cultivation of GM plants have to be prevented. Hence regulatory issues have to be stringent.

Stable nuclear transformation involving the incorporation of exogenous gene into the nuclear genome of the plant can be done by either Agrobacterial infection or biolistic gene delivery. As a result of stable gene delivery, production costs are decreased and become more simple. The exogenous proteins thus produced can be targeted to various organelles for standard eukaryotic post translational modifications. For rapid production of large amount of recombinant proteins, transient expression is the best method. One method of achieving this would be by using viral coding sequences via *Agrobacterium tumefaciens*. The other is by agro infiltration, i.e., infiltration of a suspension of recombinant *Agrobacterium* into plant tissue [1,2]. This has been specially developed as a rapid and high yield strategy for the production of clinical grade biopharmaceuticals [3,4]. Plastid transformation is another efficient alternative. The major advantage here is that the public anathema against GM plants can be reduced; the transgene cannot be transferred as pollen does not contain chloroplast [5]. High yield of recombinant therapeutic proteins have been obtained (3-6% of TSP) using tobacco chloroplast transformation [6-15].

Using biotechnology, transgenic plants have been used to produce therapeutic proteins, edible vaccines, antibodies for immunotherapy and proteins for diagnostics [16-30]. In all these cases, the therapeutics or proteins expressed in the plant tissues are either purified and used or the plant tissue is processed to a form which can either be applied topically or taken internally. Fermentors and bioreactors can be replaced with green houses with appropriate biological containment or plant growth chambers which will reduce upstream facility. Plant tissues can be processed for oral delivery of edible proteins which will reduce downstream processing too.

2. Current Status

The first recombinant plant-derived pharmaceutical protein, human serum albumin, was produced in transgenic tobacco in 1990. The concept of plant-based expression has since ex-

panded to include industrial enzymes, blood components, cytokines, growth factors, hormones, therapeutics such as antibodies, and human and veterinary vaccines [31,32] leading to federal approval (US Department of Agriculture) of a vaccine against Newcastle disease developed by Dow AgroSciences and manufactured in genetically engineered plant cells.

Literature survey over the years for plant produced antigens or vaccines describe the expression of different vaccine antigens in different plant expression systems (**Table 1**). For the commercial production of pharmaceutical products the plants chosen should express the proteins with high efficiency in a large scale. Also such systems need to gain safety and regulatory approval.

The antigen can be expressed in the cytoplasm and remain there or localized into any plant compartments like vacuole, chloroplast or Endoplasmic Reticulum (ER) by means of specific signal peptides. However the stability of the expressed antigen in the appropriate compartment has to be checked. Also, the level of protein expression for economical extraction, apparently calculated to be 1% of TSP, is very rarely realized [33].

Table 1: List of various vaccine antigens expressed in plants

Protein	Transgenic species	References
Human growth hormone	*Nicotiana tabacum*	Barta et al. 1986 (34)
Human albumin	*Nicotiana tabacum*	Sijmons et al. 1990 (35)
Human albumin	*Solanum tuberosum*	Sijmons et al. 1990 (36)
Epidermal growth factor	*Nicotiana tabacum*	Higo et al. 1993 (37)
Human interferon α	*Oryza sativa*	Zhu et al. 1994 (38)
Eritropoietin	*Nicotiana. tabacum*	Matsumoto et al. 1995 (39)
β-casein	*Solanum tuberosum*	Chong et al. 1997 (40)
α and β hemoglobin	*Nicotiana tabacum*	Dieryck et al. 1997 (42)
Human mucarinic cholinergic receptors	*Nicotiana tabacum*	Mu et al. 1997 (43)
Interleukine 2 and 4	*Nicotiana tabacum*	Magnuson et al1998 (44)
Human α 1-antitrypsin	*Oryza sativa*	Terashima et al. 1999 (45)(45)
Somatotropine	*Nicotiana tabacum*	Leite et al. 2000 (46)
Collagen	*Nicotiana tabacum*	Ruggiero et al. 2000 (47)
Lactoferrin	*Solanum tuberosum*	Chong et al. 2000 (48)
Somatotropin	*Nicotiana N. tabacum*	Staub et al. 2000 (49)
Human acetylcholinesterase	*Lycopersicon. esculentum*	Mor et al. 2001 (50)
Bovin aprotinin	*Zea mays*	Azzoni et al. 2002 (51)
Human collagen	*Nicotiana N. tabacum*	Merle et al. 2002 (52)
Lactoferrin	*Nicotiana N. tabacum*	Choi et al. 2003 (53)
Interleukine 18	*Nicotiana N. tabacum*	Zhang et al. 2003 (54)
Human granulocyte-macrophage colony stimulating factor	*Oryza sativa*	Shin et al. 2003. (55)

Epitope of C. diphthereae, B. pertussis, C. tetani	*Tomato*	Soria-Guerrra et al. 2007, 2011 (56,57)
E6 and E 7 of HPV	*Tomato*	Paz De la Rosa et al. 2009 (58)
PA of Bacilllus anthracis	*Tobacco*	Lee et al. 2011 (59)
VP2 of CPV	*Tobacco*	Ortigosa et al. 2010 (60)
FMDV	*Nicotiana. benthamiana*	Andrianova et al. 2011(61)
NP of H1N1 Influenza A virus	*Vigna uniguiculata*	Meshcheryakova et al. 2009 (62)
Hepatitis B surface antigen	*Maize*	Hayden et al. 2014, 2015 , Shah et al 2015(25,26,63)
Cholera toxin subunit B	*Maize*	Karaman et al. 2012(64)
Human epidermal growth factor	*Nicotiana. benthamiana*	Thomas and Walmsley 2014(65)
HPV type 16 L2 epitope,type 8, L1	*Nicotiana benthamiana*	Cerovska et al 2012(66) Waheed et al 2011(67,68) Matic et al 2012(69)

Stable integration, selection and maintenance of transgenic plants take time. Even then, the high level of expression is not maintained in subsequent generations. This might be due to post transcriptional gene silencing or si RNA mediated gene silencing. Expression can also be varied in nuclear transformation because of meiotic segregation. *Li et al* [70] reported the stability as well as immunogenicity of human rotavirus VP 7 protein expressed in transgenic potato for 50 generations. However this is the only report where expression study has been done for so many generations.

The steps involved in the production of recombinant proteins from plants include: (i) choice of the host species and optimization of coding sequence of the target gene in relation to the host, (ii) selection of expression cassette and creation of the expression vector, (iii) integration of the gene construct into the plant genome and regeneration of plants expressing the desired protein, (iv) identification and stabilization of the plant line for commercial production of the recombinant protein.

Selection of the host plant depends on the type of protein, i.e., the form of the recombinant protein which is to be finally used. The life cycle of the host, biomass yield, containment and scale-up costs are other deciding factors. Success largely depends upon the understanding of species- or tissue-specific factors that affect the recombinant product. Self-pollinating species are advantageous over cross pollinated plants as the spread of transgene through pollen can be reduced. This can also be addressed by using plants which can be grown in containment, e.g. Tomato which can be grown in green houses. Further, the use of plant cell cultures addresses the issue of containment where dedifferentiated cells such as in calli or cell suspensions are used and can be grown on industrial scale using fermenter.

Expression via seed specific promoters is preferred in many cases as seeds accumulate a large amount of protein, purification of proteins from seeds would also be easier. Recombinant

proteins can be stored for a longer time in storage and normally do not degrade under ambient temperature. The only disadvantage is that it takes a long time for seed set depending on the lifecycle of the plant, hence transgene expression can be assessed only then.

Several cereals including rice, wheat, barley and maize have been investigated [71,72,73] The first plant derived commercialized product was produced in maize [74,75]. Cereal plants have been adopted as a production platform by the plant biotechnology enterprises like Ventria Bioscience (http:// www.ventria.com). A rice based cholera vaccine Muco-Rice CTB was shown to be stable at RT for 8 months, as well as resistant to pepsin digestion [75]. An ETEC subunit vaccine produced in soybean seeds was found to be stable for 4 years [76]. *Hayden et al* [24,25,26] biochemically and biophysically characterized maize derived HBsAg and showed that oral delivery of wafers made from HBsAg-expressing maize germ induces long-term immunological systemic and mucosal responses.

Various parameters have to be considered and optimized for commercial recombinant protein expression in plant cells

2.1. Internal factors

a. Codon usage
b. RNA structure
c. Regulatory elements
d. Fusion protein
e. Specific tag
f. Signal peptide

2.2. External factors

1. Glycosylation
2. Proteolysis
3. Response to unfolded protein
4. Activity of chaperons
5. Compartmentalization

2.3. Product nature

1. Isolation
2. Purification
3. Storage

2.4. Different stages of transformation

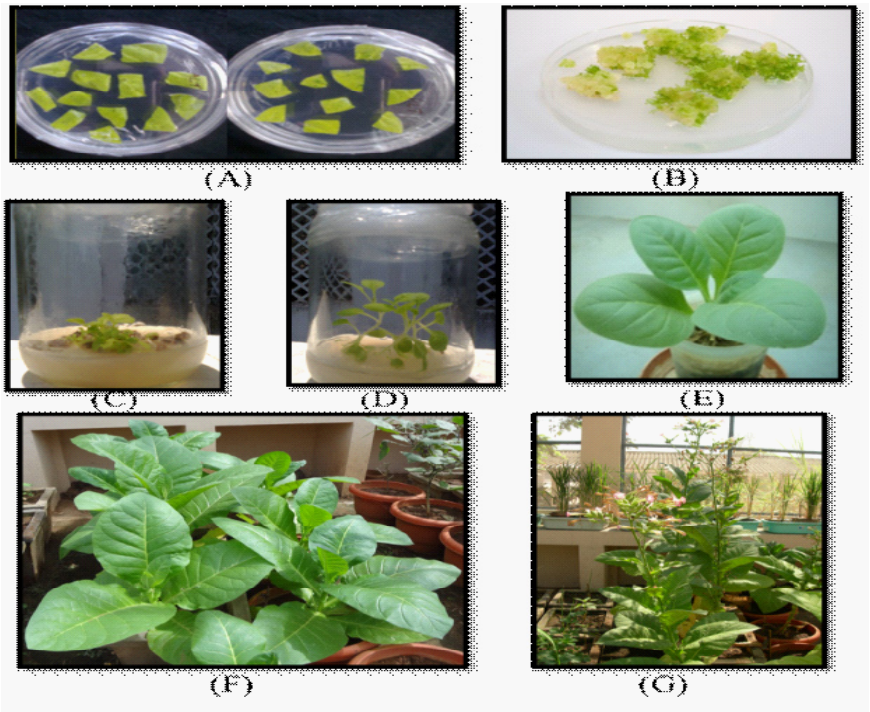

Figure 1: Different stages of transformed tobacco plant.
(A)Transformed Explants. (B) Callus induction from transformed leaflets. (C) Multiple shoots induction from callus. (D) Rooted transformed plants. (E) Transformed plant set for hardening. (F) Matured transformed plant. (G) Transformed plants-flowering.

3. A few examples where success has been achieved in recombinant protein production in Plants is mentioned below:

3.1. Plant made antibodies

There are reports of many plant produced antibodies in literature with applications ranging from diagnostics [30,77,78]; to cancer treatment [79,80,81,82,83,84]; prevention of tooth decay [86,87] ; prevention of plant disease [87,88] and preventing sexually transmitted diseases [73,90,91]. Different subclasses of antibodies (IgG, IgM) have been expressed in different plant species but *Nicotiana* species predominate [89,93].

He et al. [89] demonstrated that WNV DIII antigen (West Nile Virus) and E 16 monoclonal antibody were produced at high levels, could be purified easily and were effective in identifying WNV and also in detecting human IgM response to WNV detection. *Ganapathy et al* [30] reported the efficacy of plant produced Wb SXP1 as comparable to *E.coli* produced WbSXP1 in the diagnosis of Lymphatic filariasis, a neglected tropical infectious disease. Immunological screening using clinical sera from patients indicates that the plant-produced protein is comparable to *E. coli*-produced diagnostic antigen. These reports further substantiated that plants could serve as cost effective platform for diagnostic protein production, especially towards infectious and parasitic diseases which are prevalent in tropical countries.

Advanced plant and mammalian glycosylation differ in regard to types of sugar moi-

eties added and the type of linkages [95]. This difference in glycosylation might result in the identification of antibodies of non-human origin being seen as antigen by patients [96,129]. Plant specific glycosylation can also induce immune response. Plants now have been genetically modified to mimic typical animal glycosylation pattern by either inactivating the native enzymes or by expressing heterologous enzymes responsible for mammalian like glycosylation [97,98, 99,100].

Table 2: Examples of pharmaceutical antibodies expressed in plants

Antibody	Antigen specificities	Transgenic species	Application	References
IgG and SIgA	*Streptococcus mutans*	*N. tabacum*	Therapy (topical)	Ma *et al.* 1998(80)
IgG	Colon cancer antigen	*N. tabacum*	Therapy/Diagnostics	Verch *et al.* 1998(79)
IgG	Herpes Simplex virus HSV-2	*G. max ,O. sativa*	Therapy (topical	Zeitlin *et al.* 1998(117)
Humanized IgG	Respiratory Syncytial virus	*Z. mays*	Therapy (inhalation)	EPIcyte
IgG	*Clostridium difficile*	*Z. mays*	Therapy (oral)	EPIcyte
IgG (topical)	Sperm	*Z. mays*	Contraceptive	ReProtect LLC, MD
IgG	Hepatitis B antigen	*N. tabacum*	Therapy/Diagnostics	Valdes *et al.* 2003(118)
Diabody	Carcinoembryonic Antigen (CEA)	*N. tabacum*	Therapy/Diagnostics	Vaquero *et al.* 2002(119)
scFv	Non-Hodgkins(NHL) Lymphoma	*N. tabacum*	Vaccine	McCormick *etal. 1999,2008* 2011(120,121,122)
scFv	Carcinoembryonic Antigen (CEA	*N. tabacum P. sativum, L. esculentum, O. sativa, Triticum sp*	Therapy/Diagnostics	Stoger *et al.* 2002(91)

Two successful plant made antibodies have made to human clinical trials. Planet Biotechnology Inc. produced the world's first clinically tested antibody CaroRx™ in tobacco which specifically binds to bacteria that cause tooth decay and prevent adhesion of the organism to tooth. This is currently approved for sale as a medical device in European Union. With support from the National Institute of Allergy and Infectious Diseases (NIAID), Planet Biotechnology Inc have also developed an immunoadhesin for treatment and prevention of anthrax (PBI-220) and have tested it successfully in anthrax-infected monkeys as a therapeutic with an 80% survival rate when treatment is started after disease symptoms appear.

Middle East Respiratory Syndrome (MERS) is a recently emerged disease caused by the MERS coronavirus (MERS-CoV) endemic to the Arabian Peninsula. It has already appeared in seven Middle Eastern countries and has traveled to European countries and South Korea as well. MERS-CoV causes a pneumonia-like disease that has a fatality rate approaching

40%. Planet Biotechnology Inc have created and produced in green plants an immunoadhesin (DPP4-Fc) that has improved binding to MERS-CoV and have shown that it prevents the virus from infecting human lung cells in culture. In June 2015, the company has been awarded a Phase II SBIR grant from NIAID to support development of this candidate immunoadhesin..

In July 2008, Large scale Biology Corp reported the success of first human clinical trials testing of a plant made vaccine against cancer. A transient expression system produced patient specific recombinant idiotype vaccine against follicular B cell Lymphoma in tobacco. 16 patients immunized with their own individual therapeutic antigen showed no serious adverse effects, 70% of the patients developed cellular or humoral responses, 47% developed antigen specific response. In 2009, Bayer started the clinical development of this plant made antibody vaccine submitting Phase I study protocol to US FDA .LSBC and Bayer Crop Science also had an agreement for research and development of plant based expression of Lysosomal acid Lipase (LAL) Plant based human enzyme which could breakdown lipids, supposedly a breakthrough for Orphan diseases but this fell through and LSBC has now filed for bankruptcy.

3.2. Plant made vaccines for viral and bacterial diseases

3.2.1. Respiratory infections

3.2.1.1. Influenza is a serious respiratory disease caused by influenza viruses. It is the root cause of pandemics worldwide and prevention of influenza is a challenge as rapid mutations in subsequent generations cause antigenic variation in haemagglutinin (HA) [103]. HA is a surface glycoprotein of the influenza virus which plays a key role in viral infectivity and pathogenesis. HA is also the main target for generating protective immunity against influenza virus [104, 105]. Recent outbreaks caused by the new H1N1 swine influenza virus infected a large number of humans and raised significant concerns as a global pandemic.

The virus, A(H1N1) pdm09, which is a triple reassortant with genes acquired from swine, avian and human influenza viruses , was first detected in humans in the United States in April 2009 [105]. The highly pathogenic avian influenza A virus (H5N1) caused pandemics in poultry. Rapid evolution of the virus and easy mode of spread leads to chances of global human infection of [106]. The antigenicity of the HA protein depends on its proper folding and trimerization.It also requires multiple post-translational modifications including disulphide bond formation and glycosylation [107]. The expression of HA without its transmembrane domain from the A/Hong Kong/213/03 (H5N1) influenza virus strain fused with an ER-targeting signal at the 5’ end and the HDEL ER retention motif at the C-terminus resulted in its high-level accumulation in the ER (140 lg/g fresh weight, FW), N-glycosylation, protection from proteolytic degradation and long-term stability in Arabidopsis. Oral administration of freeze dried leaf powder expressing this HA antigen and the adjuvant saponin together elicited high levels of HA-specific mucosal IgA and systemic IgG responses in mice. It also led to the de-

velopment of neutralizing antibodies and cellular immune responses, conferring protection against a lethal viral challenge.

Normally trans-membrane domain is essential for the trimerization that is required for HA antigenicity, but plant-based HA without the transmembrane domain still could induce strong HA-specific immune responses in mice [106]. The influenza virus nucleoprotein (NP) is a highly conserved multifunctional RNA binding protein found in many different strains, making it a potential candidate for a universal vaccine. Oral immunization of maize-expressed H3N2 NP induced humoral responses in mice, showing the immunogenicity of this maize-based antigen and its potential as a universal flu vaccine candidate. The NP protein level in T1 transgenic maize seeds ranged from 8.0 to 35 lg/g of corn seed, and this level increased to up to 70 lg NP/g in T3 seeds. Cytokine analysis showed antigen-specific stimulation of IL-4 cytokines in splenocytes from mice orally administered with NP, further confirming a Th2 humoral immune response [108].

3.2.1.2. Tuberculosis (TB), caused by Mycobacterium tuberculosis (MTB), a leading bacterial infectious disease has shown reemergence and is proving to be difficult to treat due to development of drug-resistance [109]. In 2013, there were 9.0 million cases of TB, with an estimated 480 000 multidrug-resistant TB cases and 1.1 million HIV-positive individuals with more than half (56%) of these cases in South East Asia and the Western Pacific. Of the patients who suffered from the disease, 25% were in Africa, which suffered the highest rates of cases and deaths relative to the population. China and India accounted for 11% and 24% of total cases, respectively (WHO, 2014a). The 6 kDa early secretory antigenic target (ESAT6) and culture filtrate protein 10 (CFP10) proteins are among the key cell virulence factors of MTB and have been expressed in transgenic carrot plants. ESAT6 makes up <0.056% and CFP10 composes 0.002% of the total storage protein in carrot storage roots. Oral immunization of mice induced both cell-mediated and humoral immune responses [110]. Fusion of the ESAT6 antigen with other tuberculosis antigens, such as Ag85B or Mtb72F (a fusion polyprotein of two TB antigens, Mtb32 and Mtb39), and use of a transmucosal carrier such as CTB, LTB and LipY (a cell wall protein) to facilitate bioencapsulation/oral delivery, and further expression in various plant species (Arabidopsis thaliana, tobacco and lettuce), have been attempted [109,110,111].

Compared with nuclear transgenic plants, the expression levels of CTB-ESAT6 and CTB-Mtb72F in transplastomic plants reached up to 7.5% and 1.2% of TSP, respectively, increasing antigen accumulation >100 fold [109]. CTB-ESAT6 was expressed up to 0.75% of the total leaf protein in transplastomic plants. Western blot analysis of lyophilized lettuce leaves stored for up to 6 months at room temperature revealed the stability of the CTB-ESAT6 fusion protein, which retained proper folding. ESAT6 is one of the secreted proteins in the ESX-1 system, which is involved in membrane pore formation during infection. A haemolytic assay indicated the ability of chloroplast-derived ESAT6 to lyse red blood cell membranes in

a dose-dependent manner [109].

3.2.1.3. Dengue, another potential public health problem is increasing in temperate regions due to dramatic climate change. The rice codon-optimized consensus domain III of dengue virus envelope glycoprotein (E) has been fused to the M cell-binding peptide via agroinfiltration with a plant virus-based expression system. Carrying these results a step further, Kim et al [113], generated an Ebola RIC-based DENV vaccine in tobacco plants using a Gemini viral vector expression system and reported its immunogenic properties as a self-adjuvanting dengue vaccine candidate. Previously, Phoolcharoen *et al* [114] reported that plant-expressed Ebola RIC protected mice against a lethal Ebola virus challenge.

The current epidemic caused by the Ebola virus in West Africa has brought attention to a plant-produced antibody cocktail. This cocktail, called ZMAPP, was recently shown to reverse advanced Ebola disease in 100% of tested Rhesus macaques.

4. PubMed listings for therapeutic antibodies produced in plants

Antibody (generic name)	Antibody (commercial name)	Plant species	Reference
Cetuximab	Erbitux	Zea mays	Lentz et al 2012(115)
Nimotuzumab	BIOMAb EGFR, TheraCIM, Theraloc, CIMAher	Nicotiana tabacum	Rodriguez et al 2005 (116)
Palivizumab	Synagis	Nicotiana benthamiana	Zeitlin et al 2013(117)
Rituximab	Rituxan, Mabthera	Lemna minor	Gasdeska et al 2012 (123)
Trastuzumab	Herceptin	Nicotiana benthamiana	Grohs et al 2010(124)
ZMAPP		- Nicotiana benthamiana	Qui et al 2014(125)

5. Gastroenteritis and Hepatitis

Diarrhoeal infectious diseases (DID) are a major problem in developing countries, where poor sanitation prevails and food and water may become contaminated by faecal shedding [126]. Traveller's diarrhoea and cholera, caused by enterotoxigenic strains of *Eschericia coli* (ETEC) and *Vibrio cholerae*, respectively, are two enteric diseases resulting in high mortality, especially in young children [127]. CTB was expressed in maize seeds driven by a c-zein promoter and accumulated in the endosperm of transgenic maize kernels with an expression level of 0.0014% of the total aqueous soluble protein (TASP) in the T1 generation and significantly increased to 0.0197% of TASP in the T2 generation.

Anti-CTB IgG and IgA were detected in the sera and in faecal samples from orally

administered mice, and the mice were protected against CT holotoxin challenge [127]. Inclusion of a heat-stable (ST) toxin into vaccine formulations is required, as most ETEC strains can produce both LT and ST enterotoxins. Transgenic tobacco plants carrying the LTB: ST gene accumulated up to 0.05% of TSP, and oral dosing with transgenic tobacco leaves elicited specific mucosal and systemic humoral responses in mice [128]. In comparison, lettuce chloroplast-derived CTB-AMA1 and CTB-MSP1 expressed up to 7.3% and 6.1% of TSP, which is >100-fold higher expression than from the nuclear genome. CTB–proinsulin expressed up to 70% of TSP, suggesting that the fusion protein, not CTB, determines the expression level. CTB-specific antibody titres were incredibly high (up to 10 000IgA, >800 000 IgG1) and also conferred protection against CT challenge in mice, providing long-term immunity [129].

5.1. Hepatitis B virus

Hepatitis B virus attacks the liver resulting in both acute and chronic disease. Despite the availability of an effective vaccine, it still remains as a major global health problem. Hepatitis B infection causes approximately 780000 deaths every year, split up between acute hepatitis B and from liver cancer and cirrhosis due to chronic hepatitis B infection [130]. In the case of plant-derived HBV vaccines, the first report was on the expression of the small hepatitis B surface antigen (S-HBsAg) in transgenic tobacco plants. The HBsAg produced in transgenic tobacco was antigenically and physically similar to the HBsAg particles derived from human serum and recombinant yeast [131]. The yeast-derived HBsAg is clinically used for HBV vaccination. Afterward, many research groups attempted HBsAg expression in different tissues and plant species, such as tobacco, potato, lettuce, soybean, lupine, maize, tomato, peanut and Laminaria japonica. In the transgenic tobacco plant transformed with the S-HBsAg gene controlled by the 35S promoter, expression levels were very low: less than 0.01% total soluble protein and less than 10 ng/g fresh weight in leaf tissues. The expression levels of S-HBsAg in other plant species were not significantly higher; in some species, expression levels were even lower than in tobacco.

To improve vaccine production in plants, the most widely used strategies involve: (1) suitable promoters, such as strong constitutive promoters, tissue-specific promoters and promoters that are inducible by environmental factors; (2) targeting systems to specific organelles; (3) optimized codon usage; (4) alternative polyadenylation signals; (5) increased translation efficiency using leader sequences; and (6) different vector systems. Many HBsAg-over expressing transgenic plants have been developed using strong constitutive promoters, such as the 35S promoter with enhancer [138,140]. In addition to tissue-specific promoters, the patatin promoter for potato tuber [137,139], globulin promoter for maize seed [146] and fruit-specific promoters [142,143] were used. Specific organelle-, endoplasmic reticulum (ER)-, vacuole- and chloroplast-targeted strategies have also been tried [138,144].

The HBsAg has been expressed in non-edible plants, such as tobacco, using four different expression cassettes: the HBsAg gene without ER retention signal (HBS), the HBsAg gene with ER retention signal (HER), and each gene controlled by the ubiquitin promoter (UBQ) or ethylene forming enzyme promoter (EFE) [145]. The maximum expression level (19.4 ng/g FW of leaves) was observed in EFE-HBS transformed plant growth in vitro, but a higher proportion of the particulate form of the antigen was observed when it was expressed with an ER retention signal. HBsAg has also been expressed in vegetative crops, such as potato, tomato, soybean and lettuce. The expression level of transgenic potato tubers was 1–11 μg/g FW. The highest expression in a tuber was developed using a construct driven by the CaMV 35S promoter with dual enhancers, the tobacco etch virus 50-UTR, and the 30 region from the soybean vegetative storage protein gene [140]. Expression level of HBV-protein in potato was little increased when controlled by the tuber specific promoter [138].

The expression level of the major surface antigen of hepatitis B virus (P-HBsAg) reached 0.003–0.09% of TSP in transgenic potato. Mice produced specific faecal IgA and serum IgG antibodies against P-HBsAg after oral administration [140]. Herbicide-resistant lettuce was engineered to stably express the small surface antigen of hepatitis B virus (S-HBsAg) [141]. The progeny of these plants accumulated up to micrograms of antigen per gram of FW, and the S-HBsAG antigen was able to form VLPs [141]. Oral delivery of lyophilized lettuce containing low levels (100 ng) of VLP-assembled antigen with a long, 2-month interval between priming and boost administrations without adjuvant elicited both mucosal and systemic humoral anti-HB responses at the nominally protective level in mice. Lyophilized material, both as a powdered, semi-finished product or after conversion into tablets, preserved the S-HBsAg content for at least 1 year of room-temperature storage [141]. Bioencapsulated HBsAg expressed in maize reached between 0.08 and 0.46% of TSP and induced serum IgG and IgA in mice after oral administration [146]. High levels of HBsAg were obtained in maize grains, and supercritical fluid extraction (SFE)-treated maize material was used to form edible wafers. After feeding wafers containing approximately 300 lg/g HBsAg, mice showed robust serum IgG (20 000 mIU/mL) and IgA responses. Additionally, all mice administered the SFE wafers showed high sIgA and salivary IgA titres (142 mIU/mL) in faecal material, whereas Recombivax_ Merck & Co., Inc., Whitehouse Station, NJ, USA (an injected commercial vaccine)-treated mice showed no detectable titre [26]. Furthermore, mice boosted with orally administered HBsAg wafers displayed long-term memory mucosally and systemically, as evidenced by sustained faecal IgA and serum IgA, IgG and mIU/mL over 1 year [25]. Freeze-drying of S-HBsAg expressed in lettuce leaf tissue without any purification step was shown to be an important factor affecting S-HBsAg preservation. This reproducible process provided a product with VLP content up to 200 lg/g dry weight. Long-term stability tests showed that the stored freeze-dried product was stable at 4 °C for 1 year but degraded at room temperature. Animal oral immunization trials induced systemic IgG in mice (293 mIU/mL), confirming the preservation of antigenicity

and immunogenicity [161].

The biggest advantage of edible plant-derived vaccines is their easy application to oral delivery. The benefits of plant-derived edible vaccines are as follows: (1) during oral delivery, plant-derived vaccines are protected in the stomach by plant cell wall and slow release in the gut; (2) the plant tissue expressing antigen may be used as raw or dried food; (3) capsules can also be made from partially or fully purified vaccine proteins; (4) no need for cold chain systems for storage and delivery of the plant tissues or extracts; and (5) the plant-derived vaccines are cost efficient compared with traditional vaccines.

Edible plant-derived HBV antigens have been administered by oral injection or feeding in mice with/without adjuvants [138,139,140,146,148]. An oral vaccine candidate has also been administered to human volunteers in small-scale clinical trials without adjuvants. The first trial was administered to three human volunteers in row lettuce leaves in two doses (0.5–1μg of S-HBsAg/dose) without the use of an adjuvant. All volunteers responded, with two of them having serum responses in excess of the protective minimum level (10 mIU/mL of serum). However, the antibody levels declined rapidly [151,152,153]. In the second trial, previously vaccinated human volunteers were fed two or three doses of 100 g of raw potato tubers (approximately 1 mg of the S-HBsAg/dose). More than half of the subjects showed increased antibody titers [153]. The animal experiments and trials showed the potential for plant-derived HBV antigens to be used as an oral vaccine for the prevention of HBV, but there remain many problems to be solved for practical application, such as the administration of bulky plant material, declining long-term responses, individual differences in the immune response and the difficulty of defining the antigen dose [154].

The expression level of plant-derived HBV antigen is only 1/20–1/25 of the expression of yeast-derived HBV antigen; however, the expression yield and plant production scale are still increasing [154,155,156]. Tomato can be eaten without processing or cooking, hence it is an attractive candidate to develop as an oral vaccine. According to the study to date,the expression level of HBV antigen was very low as 10 ng/g FW. The maximum titers of anti-HBsAg antibody in serum is 300 mIU using oral application. This antibody yield was high compared to the expression level of HBV antigen in tomato fruits [157]. HBV antigen expression in maize produced much higher levels of antigen, and the palatability and digestibility were better than for potato. In addition, the maize system induced a strong immune response with 4632 mIU of maximum titer by both injection and oral administration [146,25]. This result suggests the possibility of providing a raw material for thermostable formulation at $0.01 per dose [146]. Plant components such as saponin, flavonoids, and plant oils also function as adjuvants [158,159,160] and help maintain the immune response in the long term [26].

The lyophilization method is an excellent way to increase the stability and shelf life of

the plant-derived vaccines. In the previous study, the storage stability of lyophilized powder form was limited at 4^{O}C [161]. In a recent study, successful long-term storage at 3_C was achieved though improvements in the process [162]. It is easier to control the concentration and standardize antigen doses and process the antigen into a tablet or capsule form using a powdered tissue instead of freeze-drying [141].

5.2. Human papillomaviruses

Cervical cancer caused by HPV infection is the fourth most common cancer among women worldwide and has become a global concern, particularly in developing countries, which bear approximately 80% of the burden [163]. Furthermore, HPV type 16 is by far the most prevalent type and is correlated with 54% of cervical cancer cases [164]. Higher levels of specific IgG and IgA levels (<1 : 1000 for the L1/LT-B group and <1 : 500 for the L1 group) of HPV-16L1 (major capsid protein) were induced when mice were immunized with transgenic tobacco-derived HPV-16L1 combined with LTB by the oral route [165]. A novel HPV 16L1-based chimericvirus-like particle (cVLP) expressed in tomato plants contains a string of T-cell epitopes from HPV-16 E6 and E7 fusion at the C terminus.

Long-lasting specific IgG antibodies with neutralizing activity were detectable for 12 months after induction by immunization with cVLPs. Efficient long-term protection and tumour growth inhibition were elicited by TC-1 tumour cells expressing HPV-16 E6/E7 oncoproteins, whereas significant tumour reduction (57%) was observed in mice administered with these cVLPs [166].

5.3. Rabies

Rabies virus is an enveloped, negative-sense, single-stranded RNA virus of the genus *Lyssavirus* in the family *Rhabdoviridae*. This zoonotic disease causes acute, progressive, incurable viral encephalomyelitis and is usually transmitted through the bite of an infected animal, resulting in 40000–100000 human deaths annually worldwide [167]. The expression level of the rabies virus glycoprotein protein (G protein) in transgenic maize kernels reached 25 lg/g FW. Neutralizing antibodies in sheep were induced after oral immunization with maize-derived G protein. Further, the degree of protection achieved with 2 mg of maize-based G protein was comparable to that of a commercial vaccine [168]. Transgenic hairy roots of Solanum lycopersicum were engineered to express the rabies glycoprotein fused with ricin toxin B chain (rgp-rtxB) antigen driven by a constitutive CaMV35S promoter. The expression level of the RGP-RTB fusion protein in different tomato hairy root lines ranged from 1.4 to 8 lg/g of tissue. A partially purified RGP-RTB fusion protein was able to induce an immune response in BALB/c mice after intramucosal immunization, but the IgG titres were low [169].

5.4. Malaria

Malaria is a mosquito-borne infectious disease caused by Plasmodium parasites [170]. Despite decades of intensive research efforts, at present there is no vaccine that provides sustained sterile immunity against malaria. In this context, a large number of targets from the different stages of the *Plasmodium falciparum* lifecycle have been evaluated as vaccine candidates. None of these candidates has fulfilled expectations, and as long as we lack a single target that induces strain-transcending protective immune responses, combining key antigens from different lifecycle stages seems to be the most promising route toward the development of efficacious malaria vaccines. *Plasmodium falciparum* is responsible for the majority of the over half a million malaria deaths per year, which are predominantly children under the age of five that live in indigent African nations [171]. A chloroplast-derived dual cholera and malaria vaccine expressing CTB fused with the malarial vaccine antigens apical membrane antigen 1 (AMA1) and merozoite surface protein 1 (MSP1) accumulated up to 13.17% and 10.11% of TSP in tobacco and up to 7.3% and 6.1% of TSP in lettuce, respectively. The AMA and MSP titres were lower than those of CTB, suggesting that the CTB antigen could saturate the immune system. Significant levels of antigen-specific antibody titres in orally immunized mice not only cross-reacted with the native parasite proteins in immunofluorescence studies and immunoblots, but also completely inhibited the proliferation of the malarial parasite [172]. Oral immunization of mice with the MSP1 and circumsporozoite protein (CSP) fusion protein (MLC) chimeric recombinant protein expressed in *B. napus* successfully elicited antigen-specific IgG1 production. Th1-related cytokines interleukin 12 (IL-12, a cytokine involved in the differentiation of naive T cells into Th1 cells), TNF (tumour necrosis factor, a cytokine involved in the inflammatory process and apoptosis) and IFN-c were significantly increased in the spleens of immunized mice [173].

Spiegel *et al* [174] demonstrated the use of a plant transient expression platform based on transfection with *A.tumefaciens* as essential component of a malaria vaccine development workflow involving screens for expression, solubility and stability using fluorescent fusion proteins.

6. Plant Made Pharmaceutics for Veterinary Use

Vaccination based on the programming of the specific mechanisms of warm-blooded animal protection against pathogens is the most efficient method for the struggle against infectious diseases, which often result in a mass mortality. In agriculture, there is no alternative to livestock vaccination, because there are no anti viral drugs that are suitable for a wide use in animal husbandry. The importance of animal vaccination indirectly affects human health, because the use of vaccines significantly reduces the amount of pharmaceuticals in the food chain.

As a rule, animal immune mechanisms are activated by the direct introduction of infectious agents or their components. At present, most of used vaccines are preparations on the basis of inactivated agents. Although these vaccines manifest the high immunogenicity, they are not without serious shortcomings. Among such disadvantages is the increased sensitivity of the organism to them, the large load on the immune system, the reactogenicity of vaccines (side effects), their toxicity etc. Manufacturing of medicinal preparations for veterinary use is a very important and dynamically developing field of world industry.

Pathogen	Antigen	Plant	Reference
Group A rotovirus of cattle	Protein VP6 Protein VP4	Solanum tuberosum cv mayqueen Nicotiana benthamiana S.tuberosum cv Bintje Medicago sativa	Matsumara et al 2002(175) O'Brien et al 2000(176) Yu et al 2003(177) Wig dorovitz et al 2004(178)
Bovine Herpes Virus BHV 1	Glycoprotein	N.benthamiana	Perez et al 2003(179)
Foot and mouth disease	Protein VP1 VP1 and VP7	M.sativa, Chenopodium quinoa,N.benthamiana, Stylosanthes guianinses cv Reyan II	Wigdorovitz et al 1999(178) Wang et al 2007(180) Yang et al 2006(181)
Rinderpest virus of cattle	Haemagluttinin H	N.tabacum, Arachis hypogaea	Khandelval et al 2003, 2004(182,183)
Canine parvovirus	Peptide from protein VP2	Arabidopsis thaliana	Gil et al 2002(184)
Newcastle disease virus of birds	Proteins F and NH Glycoprotein F	*S. tuberosum* cv. Kennebec *Z. mays* *Oryza sativa*	Beristine 2006(185) Yang 2007 (187) Guerrera 2007(186)
Avian influenza H5N1	Haemagglutinin M2 Haemagglutinin rHA0	N.benthamiana	Nemchinov et al 2007(188) Kalthoff et al 2011(189)
Japanese encephalitis virus(horses, cattle, pigs)	Glycoprotein E	O.sativa cv Nipponbare	Wang et al 2009 (190)
Rabies	G protein G and N proteins G protein N protein G protein	*L.esculentum*var.UC 82b *N.benthamiana,Spinacea oleracea,N.tabacum cvSamsun,Zea mays* *D carota var.Rendidora*	McGarvey et al1999(191) Modelska et al1999(192) Yusibov et al2002(197) Loza-Rubio et al 2008, 2012 (132,168) Perea-Aranga et al 2008(195) Rojas-Anaya et al 2009(196)

Dow Agrosciences in 2006 received the first regulatory approval for plant made vaccine against Newcastle Disease Virus from USDA. As a part of the approval process, USDA verified that the plant produced protein is equivalent to other Newcastle vaccines. This vaccine

was composed of recombinant hemagglutinin neuraminidase expressed in transgenic tobacco suspension cells Although this never came forward as a commercially available product, the formulation advanced through USDA Center for Veterinary biologics regulatory approval. Hernandez et al [198] had also showed the efficacy of orally delivered papaya produced anti-cysticercosis vaccine and its potential as a low cost alternative of immunization. Major et al [199] showed that intranasal vaccination with a plant-derived H5 NA vaccine protected mice and ferrets against highly pathogenic avian influenza virus challenge. An edible potato based vaccine has also been developed against chicken infectious bronchitis virus [200]. Chicken were immunized by oral delivery of sliced tubers expressing S1 glycoprotein in doses over two weeks. These immunized birds developed virus specific antibody response and were protected against IBV. Another research group succeeded in vaccinating chickens against infectious bursal disease virus (IBDV) with plant made VP2 protein. Chicken orally immunized with Arabidopsis crude leaf extracts or transgenic rice seeds were protected to a similar level achieved with a commercial injectable vaccine [201]. Recently, the VP2 antigen was produced transiently in Nicotiana benthamiana and induced neutralizing antibodies in immunized chicken [202].

Transgenic maize seeds expressing envelope spike protein of Transmissable gastroenteritis corona virus (TGEV) were seen to raise neutralizing antibodies in piglets. This antigen was also stable during various stage conditions [203]. Enterotoxigenic E. coli (ETEC) causing post-weaning diarrhea in piglets has been a target for a plant-made vaccine. The major subunit protein of ETEC F4 fimbriae has been expressed in the leaves of tobacco [204], alfalfa [205] and in seeds of barley [205]. This subunit vaccine was shown to be immunogenic and partially protective after oral delivery to weaned piglets [205].

7. Veterinary Vaccines in Various Stages of Clinical Trial

Product	Disease	Plant material	stage	Developer
Fused proteins containing rabies virus epitopes	Rabies	Spinach	Phase 1 over	Thomas Jefferson University
HN Protein of NDV	NDV of birds	Tobacco cell suspension	Approved by USDA	Dow AgroSciences
Mixture of antiviral vaccines	Diseases of horses, dogs and birds	Tobacco cell suspension	Phase 1	Dow Agrosciences
Vaccine for Birds	Coccidiosis	Modified rape plants	Phase 2	Guardian Biosciences, Canada

8. Plant Made Therapeutics for Human Use

There have been many reports of therapeutics expression in plants including anticoagu-

lants [207]; thrombin inhibitors [207] ; HIV [208]; Diabetes [209]; Liver cirhosis and burns [210]; Hepatitis [207,211]; anemia [210]; hemophilia [212]; organophophate poisoning [207]; Hypertension [213] etc. Shenoy et al [214] reported that the oral delivery of Angiotensin-Converting Enzyme 2 and Angiotensin-(1-7) bioencapsulated in plant cells attenuates pulmonary hypertension. Further this also provided proof-of-concept for a novel low-cost oral ACE2 or Ang-(1-7) delivery system using transplastomic technology for pulmonary disease therapeutics.

Taking advantage of the high number of chloroplast genomes per cell, Daniell's group optimized technology for chloroplast transformation and gene expression. Oral administration of factor VIII or FIX antigens expressed in transplastomic tobacco plants suppressed inhibitor formation and anaphylaxis in hemophilic mice. A combination of protection from digestion offered by bio encapsulation in plant cells and fusion to the transmucosal carrier cholera toxin B (CTB subunit, thereby targeting gut epithelial cells) resulted in efficient tolerogenic delivery.

The first plant made therapeutic to reach phase II human trials, Locterin, by Biolex therapeutics, was a plant made controlled release interferon alpha treatment for chronic hepatitis [211]. First plant made therapeutic to reach phase III trials was a therapeutic developed by Protalix BioTherapeutics against Gauchers disease expressed in carrot suspension cells [213]. Human cereberoside expressed by carrot cells (human pr GCD) had high batch to batch enzymatic activity. In December 2009, Pfizer and Protalix entered an agreement to develop and commercialize pr GCD. However in early 2011, FDA declined the approval of the drug asking for additional data from existing studies, but not asking for additional trials.

U.S. Food and Drug Administration granted approval for ELELYSO, a product of Protalix Biotherpeutics and Pfizer for injection in May 2012 as a hydrolytic lysosomal glucocerebroside-specific enzyme ELELYSO™, which is branded as UPLYSO (Taliglucerase alpha) in Latin America, which is a plant cell-expressed form of the glucocerebrosidase (GCD) enzyme. This enzyme is indicated for long-term enzyme replacement therapy (ERT) for adults with a confirmed diagnosis of Type 1 Gaucher's disease. Approvals have also been granted by the applicable regulatory authorities in Uruguay, Mexico, Australia, Canada, Chile and other countries. (www.protalix.com) Sembiosys has also completed Phase I and II trial of safflower produced insulin grown in seed bioreactor Using Seed crops, ORF Genetics also produces various growth factors and cytokines in transgenic barley for use in cosmetics.

9. Against Helminths and Protozoans

Toxoplasma gondii, an intracellular parasitic protozoan can cause complications in pregnant women and in immunodeficient individuals like HIV positive patients and organ transplant recipients [215]. Recent studies have shown that chronic toxoplasmosis infection can play major role in the aetiology of certain mental disorders, such as schizophrenia [216].

Expression of the T. gondii dense granular protein 4 (GRA4) antigen via chloroplast transformation (chlGRA4) led to its accumulation to approximately 6 lg/g FW (0.2% of total protein) in tobacco plants. Oral immunization with chlGRA4 elicited both mucosal and systemic immunity (<1000 IgG titre) and also showed a 59% decrease in the brain cyst load of mice. Chloroplast-derived GRA4 elicited a protective immune response against Toxoplasma infection by reducing parasite loads in mice, correlating with a mucosal and systemic balanced Th1/Th2 response [217]. Toxoplasma gondii main surface antigen (SAG1) fused with the 90-kDa heat-shock protein from Leishmania infantum (LiHsp83) as a carrier expressed in transplastomic tobacco plants reacted with human seropositive samples in a functional analysis. Oral immunization with chLiHsp83-SAG1 also induced a significant reduction in the cyst burden in mice, which correlated with an increase in specific anti-SAG1 antibodies [218].

Cysticercosis, an endemic parasitic disease caused by Taenia solium, affects human health and the economy in developing countries. Cysticercosis cysts in the central nervous system produce neurocysticercosis (NCC), a common cause of acquired epilepsy [219]. The S3Pvac vaccine components (KETc1, KETc12, KETc7 and GK1 [KETc7]) and the protective HP6/TSOL18 antigen were expressed using a Helios2A polyprotein system through the 'ribosomal skip' mechanism. The 2A sequence (LLNFDLLKLAGDVESNPG-P) derived from the foot-and-mouth disease virus induces self-cleavage events at the translational level, releasing the distinct antigens in a single transformation and expression event. Plant-derived Helios2A accumulated up to 1.3 lg/g FW in transgenic tobacco leaf tissue and was recognized by antibodies in the cerebrospinal fluid from patients with NCC in a functional assay. Further, orally immunized mice elicited an immune response, but antibody titres were not reported [220].

Lymphatic filariasis caused by Brugia malayi, Brugia timori and Wucheraria bancrofti though not fatal; still is the second leading cause of permanent and long term disability worldwide. Filariasis has a spectrum of disease manifestation and infectivity found among the infected individuals; it also goes unnoticed for years. In areas where it is endemic, humans become infected in early life and are persistently infected by the presence of adult worms in their lymphatic system. The ability to survive long term in the hostile mammalian system shows that the parasite has evolved evasion mechanisms to avoid being attacked by the mammalian immune system. Ganapathy et al [29] reported the transformation of N tabacum with Brugia malayi Abundant Larval Transcript-2 (Bm ALT-2), a major antigen produced from recombinant E.coli found to be experimentally successful as potential vaccine candidate against lymphatic filariasis. The level of expression varied from 50 to 90 ng/lg of total soluble protein for ALT-2. Immunization of mice with plant-extracted protein indicated that the plant-produced protein had immunological characteristics similar to the E.coli-produced protein. Antibody titers produced by plant produced recombinant ALT 2-immunized mice were on par with those immunized with recombinant protein produced by E.coli. Antibody isotype assay showed that

plant-produced recombinant ALT-2 induced significant IgG1, whereas E.coli-produced recombinant ALT-2 induced IgG3.

The same group also reported the expression of WbSXP-1, a diagnostic antigen for the easy detection of lymphatic filariasis, isolated from the cDNA library of L3 stage larvae of Wucheraria bancrofti, in tobacco plants [30]. The immunoreactivity of the plant-produced WbSXP-1 was assessed based on its reaction with the monoclonal antibodies developed against the E. coli-produced protein. Immunological screening using clinical sera from patients indicates that the plant-produced protein is comparable to E. coli-produced diagnostic antigen

10. Challenges

The selection of the host plant depends upon the nature ot the recombinant protein. Hence depending on the protein, a suitable plant would have to be chosen and strategies would have to be optimized in that plant for maximum yield. Thus development of a common plant platform where any protein can be expressed is not possible . The selection of the suitable host plant also has to be based on economic issues like storage property, scalability of protein production after opimization of the conditions, transportation, cost effectiveness of downstream processing, preferably short timescale in production and also edibility. Efficient transformation and regeneration protocols would also be a criteria to choose a suitable plant host. Some recombinant proteins might also affect the natural metabolic pathway in plants or cause toxicity to natural plant proteins. It can also retard plant growth or reduce the production of some key roteins which are required for normal plant function. These toxic side effects can be decreased by the identification of intermediate metabolites involved in toxicity and altering them or targeting them to organelles.

11. Safety Issues

Production and wide distribution of biopharmaceuticals is hindered by a number of circumstances. The first of them is related to the problem of biosafety – the cultivation of genetically modified plants in the field can lead to the accidental introduction of foreign genes into crops grown for human consumption. Therefore, companies producing biopharmaceutics focused on plant species, which are absent from the food chain of humans and animals and also on growing of genetically modified plants preventing their crosspollination with other crops. The second difficulty is related to the necessity of plant material treatment for the removal of various undesired compounds, such as lignin, proteases, phenolic compounds, and pigments, especially in the case of plant species, which are not consumed. All these result in the requirement of additional studies. The third circumstance is due to the fact that until now all aspects of maintaining and growing of plants producing biopharmaceuticals are not settled at the legislative level.

Biosafety issues can be covered by the application of chloroplast transformation and/or growing the plants in contained facilities [221,222]. Furthermore, an inducible system can be used to control the transgene expression when required. Transgenic plants can be grown at the site where the vaccine is needed. This advantage can save the costs related to transportation and cold storage. Plant-derived vaccines have the potential to be used as oral vaccine, thus evading the costs related to sterile needles and trained medical staff.

11.1. Stability

Plants-derived vaccines are likely to be more stable. A recent report shows that a chloroplast-derived vaccine candidate was stable at room temperature for 20 months [223]. Moreover, mice immunized with the vaccine stored at room temperature showed similar IgA/IgG levels as those of mice immunized with the vaccine stored at 4°C. This characteristic is very important for the development of a vaccine for developing countries where cold chains are difficult to maintain in remote areas.

12. The Future

Though the benefit of plant made pharmaceuticals have been pointed out reportedly it is being implemented only now due to investment by big pharmaceutical companies. Plant based systems have been able to reproduce a wide variety of human proteins, including those that have multiple subunits expressed and assembled in plants as well as proteins and vaccines requiring Co expression of additional modifying enzymes. While raw edible vaccines are unfeasible for human therapy, it may not be necessary to fully isolate the target protein from plant material. A middle ground of dried and ground plant material may be more suitable for oral delivery of some vaccines and therapeutics. This would be an excellent option for the production of veterinary medicines where recombinant feed could contain vaccine antigens and would be useful and cheap for developing nations [224,225]. If yields can be standardized, there is potential for delivery of therapeutics in unprocessed plant material, especially in veterinary field where the dosage has a wide active range. The use of vaccines and prophylactics for the control of infectious diseases in the livestock industry will grow as antibiotics applications diminish. Plants as bioreactors comprise a valuable option for production of recombinant protein therapeutics for animal health. In recent years numerous studies demonstrated the feasibility and advantages of plant-based production platforms for various proteins with therapeutic use, including complex antibodies, subunit vaccines and immunogenic virus-like particles. Plant made therapeutic products are currently on the cusp of widely entering biotechnology markets. Interaction and concerted actions of the plant biotechnology sector with veterinarians and regulatory authorities will facilitate development of novel approaches to sustainable, antibiotic-free livestock agriculture.

13. Acknowledgement

Research reported from the authors laboratory was funded by WOS –A Scheme Of DST, Govt of India for MG and ICMR Emeritus Medical Scientist grant to PK. The authors wish to thank Director, Centre for Biotechnology, Anna University for additional support.

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