Advanced Techniques in Biology & Medicine

ISSN: 2379-1764

Ganapathy, Adv Tech Biol Med 2016, 4:1
DOI: 10.4172/2379-1764.1000161

Mini Review Open Access

Plants as Bioreactors- A Review

Mathangi Ganapathy*

Centre for Biotechnology, Anna University, Chennai, Tamil Nadu, India

Abstract

Currently available systems for the commercial production of recombinant subunit vaccines include bacteria, yeasts, insects and mammalian cell cultures. Each of these systems has specific benefits, but overall, their application is limited by insufficient scalability, cost, safety and target integrity. Plant-based production platforms remains attractive as an alternative due to high scalability, cost-effectiveness and greater safety. Vaccines have been developed against viral, bacterial, parasitic and allergenic antigens for human as well as animal use using plant expression systems. Stable integration of transgene into the nuclear or chloroplast genome in many plants (e.g. tobacco, tomato, potato, papaya, carrot) for the production of subunit vaccines have been reported, effective expression has also been achieved by transient expression. Many plant produced recombinant proteins have shown immunogenicity, several have been shown to work effectively in animal models. This review tries to give an update of plant produced recombinant proteins, the future and limitations.

Keywords: Plant produced recombinant proteins; Plant produced pharmaceutics; Gene transformation; Transplastomic technology; Vaccine; Immunological response

Ever since the discovery of genetic manipulation and the establishment of various methods for effective transformation of cells, the use of plants as an expression system for the production of recombinant therapeutic proteins have been advocated. This has been an exciting as well as controversial concept. 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 as the problem caused by illegal or unethical cultivation. 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. Decreased costs and simplification of production process are the results of stable gene delivery. 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 biotechnological approach, 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 can be 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.

In spite of more than twenty years of research and reports about the efficacy of plant produced vaccine related products very few products have gone all the way through the production and regulatory hurdles [31].

Current Status

Literature survey over the years for plant produced antigens or vaccines describes 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 types of plants and the types of plant tissue used for the production of therapeutic proteins include leaf and stem tissues of various species and varieties of tobacco (*N. benthamiana, N. tabacum*), *Arabidopsis thaliana* [32-34], alfalfa [35-37] and potatoes [38-41], seeds of rice, beans, maize and tobacco [42-45]; root vegetables like carrots [46,47], fruits like tomatoes and strawberries [48-54]; aquatic weeds like *Lemna* sp [55,56]; hairy root cultures derived from various plants via *Agrobacterium rhizogenes* transformation [57-59]; single cell cultures of the algae Chlorella [60,61] and Chlamydomonas [62,63]; suspension cell cultures of tobacco [64-67]; transformed chloroplasts of a variety of plant species [68-70].

Transgenic single-cell cultures offer the advantages over whole plant systems of a high level of contaminant and possibility of producing proteins in bioreactors under Good Manufacturing Conditions (GMP)

***Corresponding author:** Mathangi Ganapathy, Centre for Biotechnology, Anna University, Chennai, Tamil Nadu, India, Tel: 91-9444336040; E-mail: mathangig@gmail.com

Received December 28, 2015; **Accepted** January 11, 2016; **Published** January 18, 2016

Citation: Ganapathy M (2016) Plants as Bioreactors- A Review. Adv Tech Biol Med 4: 161. doi: 10.4172/2379-1764.1000161

Epitope source	Plant host	Reference
DPT polypeptide of *C. diphthereae, B. pertussis, C. tetani*	Tomato	Soria-Guerrra et al. [51,188]
E6 and E 7 of HPV	Tomato	Paz De la Rosa et al. [189]
PA of *Bacilllus anthracis*	Tobacco	Lee et al. [190]
VP2 of CPV	Tobacco	Ortigosa et al. [191]
FMDV	*N. benthamiana*	Andrianova et al. [192]
NP of H1N1 Influenza A virus	*Vigna uniguiculata*	Meshcheryakova et al. [193]
Hepatitis B surface antigen	Maize	Hayden et al. [25-27]
Cholera toxin subunit B	Maize	Karaman et al. [194]
Human epidermal growth factor	*N. benthamiana*	Thomas and Walmsley [24]

Table 1: Some examples of vaccines, therapeutic proteins expressed in plants.

conditions, as in currently the case with conventional fermentation or cell culture techniques.

Generally the use of plants for production of therapeutic proteins means a lower cost of production and easier expansion for large volume production than cell culture systems. Plant expression systems can potentially produce hundreds of kilograms per year of a purified protein whereas the cost of a similar production capacity using mammalian cell cultures may be prohibitive [71].

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].

Stable integration, selection and maintenance of transgenic plants take time. Even when it has been achieved, 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. Also in the case of nuclear transformation expression can be varied because of meiotic segregation. Li et al. [72] 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. This seems to be an area where much work has not been done.

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, 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 [73].

Green leaf expression or constitutive expression is easy to achieve and the rate of protein expression would be high. One of the major disadvantages associated with the leafy crops is their limited shelf life, hence immediate processing of the harvested material has to be done. Also purification strategies would have to be optimized to separate the protein of interest from leaf constituents like pigments, alkaloids and other secondary metabolites.

Seeds accumulate a large amount of protein during developmental stage, hence expression via seed specific promoters is preferred in many cases, this also has the advantage that purification would be easier. Also proteins can be stored for a longer time in storage and proteins 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 [74,75].The first plant derived commercialized product was produced in maize [76,77]. 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 [78]. An ETEC subunit vaccine produced in soybean seeds was found to be stable for 4 years [79]. Recent publication from Hayden et al [26] shows that oral delivery of wafers made from HBsAg-expressing maize germ induces long-term immunological systemic and mucosal responses.

Oil crops offer unique production advantages of inexpensive downstream processing to obtain the desired proteins if they are targeted to the oil bodies. Oleosin, a plant protein, is present on the oil body surface. The hydrophobic central core part of the oleosin remains inserted into the oil body while the amphipathic and less conserved N and C-terminals reside on the surface [80]. The protein in question can be targeted to oil bodies as an oleosin fusion which can be later removed by centrifugation-based methods that separates oleosin fused protein from most of the other contaminants [81]. The oil bodies and proteins associated with them can be easily separated from the majority of other seed cell components by floatation centrifugation, taking advantage of their low density. To facilitate the recovery of pure protein, a specific protease cleavage site can be inserted between oleosin and the desired recombinant protein. An example of oil crop being utilized for this purpose is the oleosin fusion platform that has been developed by SemBioSys Genetics Inc. (http://www.sembiosys.com/), where the recombinant proteins are targeted to oil bodies of safflower seeds. Thrombin inhibitor, hirudin, was produced in transgenic seeds of *Brassica napus* using this strategy .The engineered oil bodies with their associated proteins can be used as affinity matrices for the selective, non-covalent binding of desired target molecules. For this, the oil-body proteins may be genetically fused to a ligand having specificity for the desired target molecule [82]. The expression of recombinant protein

as translational fusion with oleosin protein exposes the recombinant protein to cytosol, but at the same time it protects the foreign protein from cytosolic degradation. Because the fusion protein is not exposed to the environment of ER lumen, it avoids the posttranslational modifications.

The use of moss as a bioreactor is one of the major innovations in the manufacturing of biopharmaceuticals, which is cost effective and at the same time, avoids risks associated with environmental release of transgene. Moss allows humanization of glycosylation patterns and also time taken to reach the market is comparable to traditional systems. A transient expression system allows feasibility studies within 10–12 weeks and stable production strain development takes 4–6 month. Cultivation in suspension allows up-scaling of the photo bioreactors up to several 1000 L. Heterologous proteins are secreted into the medium. Downstream processing from this low salt medium involves fewer purification steps. They have high vegetative growth rate which shortens the production cycle. Genome engineering is greatly facilitated by the availability of Physcomitrella genome sequence. Facilities for production under GMP standards as well as long term storage are being processed [83-85].

Other aquatic plants and green algae (Chlamydomonas, Wolffia, Spirodela, Chorella, etc.) can also be used for the production of recombinant proteins. The potential of *C. reinhardtii* as a bioreactor has been demonstrated by the expression of hGAD 65 against type I diabetes [86], D2 fibronectin domain *of Staphylococcus aureus* as a fusion protein with CTB under the control of rbcL UTRs which provided 80% protection against lethal challenge in immunized mice [87]. Further, these can be grown under containment. Aquatic plants and green algae can be an alternative to open field plants [88-90].Though expression levels are highly variable by gene, improvements in codon optimization [91,92] and characterization of gene regulatory elements [93,94] combine to increase the level of transgene expresssion. *C. reinhardtii's* success as a future platform for the production of recombinant proteins has been reviewed [95].

Plant suspension cultures can be used to express the recombinant proteins. Many reports show how plant cells can produce diverse pharamaceutical proteins such as cytokines, growth regulators, nutraceuticals, etc. [96-98]. Some of these products have entered clinical development others are used in diagnostics, research or veterinary applications. Close to market products include gastric lipase for the treatment of cystic fibrosis, (Meristem therapeutics France), glucocerebrosidase for the treatment of Gaucher's disease (Protalix Biotherapeotics, Israel), anti-inflammatory molecules like lactoferrin, lysozyme (Ventria, USA). The first plant derived peptide likely to reach market is insulin produced in Safflower seeds by the Canadian company SemBioSys Genetics (http://www.sembiosys.com).

To achieve high level of transcription, the strength and expression profile of the key regulatory element "promoter" which drives the transcription, play an important role. The promoters that induce the expression of genes irrespective of the environment or developmental factors are called as constitutive promoters. They are generally used for the production of recombinant proteins in all the tissues of a plant. The cauliflower mosaic virus 35S promoter [99] has been used extensively for this purpose and high-level expression of recombinant proteins has been achieved [100]. It is more effective in dicots than monocots probably because of the differences in quality/quantity of regulatory factors. CaMV35S promoter has been used to produce several antigenic proteins in plants including CTB, LTB, HBsAg protective antigen, rabies virus glycoprotein G, SARS virus glycoprotein S [101-107] and other products of therapeutical antibodies, spider silk, SMAP-29 peptide, streptavidin, avidin and adiponectin [108-114].

Ubiquitin promoter is another most commonly used constitutive promoter Stoger et al. transformed rice with the gene encodingscFvT84.66 under the control of maize ubiquitin promoter or enhanced CaMV35S promoter. The expression levels of recombinant antibody were found to be comparable in the leaf tissue using either promoter. Using ubiquitin promoter, expression levels in the leaf tissue and seeds were comparable, but recombinant protein was not detected in seeds of transgenic plants when CaMV35S promoter was used [115]. Several molecules including CTB, LTB, HBsAg, human interferon, avidin or aprotonin have been produced in plants using ubiquitin promoter [116-121]. Generally, transgene expression varies in the transgenic plants generated using the same construct, even in the same environment. It may be because of position effect, transgene copy number or silencing [122-127]. There is a possibility to engineer desirable elements in the expression cassette to obtain the uniform expression levels.

Nuclear matrix attachment regions (MAR), also called global regulatory sequences, that are thought to influence gene expression, can be used to enhance the transcriptional activity of the transgene. They are supposed to place the surrounding loci in the regions which are suitable for recruitment of transcription factors to promoters [25,26]. Further, these AT-rich elements have been shown to reduce position effect by forming chromatin loops and therefore, increase transgene expression [127-131]. Also these have been found to maintain the expression level in subsequent generations [132].

Once the protein is synthesized, it undergoes several modifications before final delivery to its target. These modifications include glycosylation, phosphorylation, methylation, ADP-ribosylation, oxidation, acylation, proteolytic cleavage involving the polypeptide backbone and non-enzymatic modifications like deamination, glycation, racemization and spontaneous changes in protein confirmation [133]. The glycosylation machinery of ER is conserved in most of the species and adds similar glycans that belong to oligomannose category [134,135]. Yeast, insects, mammals and plants attach high-mannose glycans to the same Asn residue in the ER, but they differ in trimming and further modification of the glycans in the Golgi apparatus. Plants have the capacity to add complex N-linked glycans with a core substituted by two N-acetylglucosamine residues which is similar to the glycosylation pattern observed in mammals [136]. However, plants do not add galactose and terminal sialic acids but add plant-specific α-(1,3)- fructose and β(1,6)-xylose residues ,which are not desirable [137,138]. Various strategies have been developed to humanize the glycan patterns generated by transgenic plants.

Plant made vaccine

Dow Agrosciences in 2006 announced that it had 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. Although this never came forward as a commercially available product the formulation advanced through USDA Center for Veterinary biologics regulatory approval. Hernandez et al [139] had also showed the efficacy of orally delivered papaya produced anticysticercosis vaccine and its potential as a low cost alternative of immunization. Major et al [140] showed that intranasal vaccination with a plant-derived H5 HA vaccine protected mice and ferrets against highly pathogenic avian influenza virus challenge. Protective antigens of multiple strains of

influenza have been transiently expressed in *N. benthamiana* using an Agrobacterium mediated transient expression system. The agro-infiltrated plants produced large amounts of protective antigen from H5N1 (AIV) and H1N1 (human) strains [141,142]. The production of this antigen was performed in less than 3 weeks from the release of viral sequence to purified product. This plant made influenza vaccine has completed Phase II human trails. Mortimer et al [143] has reported the development of a plant-based platform for producing influenza vaccines locally, in South Africa. This was done with an idea to set up platforms in developing countries for vaccine production which would be helpful in times of pandemics.

Recently Hayden et al [26] compared the long term immunological memory against HBsAg the hepatitis B surface antigen in mice injected with a primary dose of Recombivax® and boosted with orally-delivered HBsAg maize germ wafers, control wafers, or parenterally-delivered commercial vaccine (Recombivax®). They have reported that mice boosted with HBsAg orally administered wafers displayed increase in mucosal IgA titres in fecal material and steep increase in in serum IgA whereas mice boosted with Recombivax showed no detectable levels of IgA. They have concluded that oral delivery can provide long term immunoresponses mucosally and systemically. This was a follow up paper from the same group which had reported earlier that oral feeding of supercritical fluid extraction treated maize material to mice elicited robust IgG and IgA responses which was comparable to injected commercial vaccine, Recombivax® [25]. Latest publication from the same group have reported the biochemical and biophysical characterization of maize-derived HBsAg and established that SFE-treated maize material is a viable oral vaccine candidate and this major advancement in the development of the first oral subunit vaccine [27].

Plant made antibodies

There are reports of many plant produced antibodies in literature with applications ranging from diagnostics [30,144-147]; to cancer treatment [148-153]; prevention of tooth decay [154-156]; prevention of plant disease [157,158]; and preventing sexually transmitted diseases [75,114,159]. de Muynck [160] has given a comprehensive review about this. Different subclasses of antibodies (IgG, IgM) have been expressed in different plant species but *Nicotiana* species predominate [161,162].

He et al. [163] 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 *Wb*SXP1 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 moieties added and the type of linkages [164].This difference in glycosylation might result in the identification of antibodies of non-human origin being seen as antigen by patients [165,166]. 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 [160,167-169].

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 undergoing Phase II clinical trials.

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.

Additional therapeutic proteins

There have been many reports of therapeutic s expression in plants including anticoagulants [170]; thrombin inhibitors [170]; HIV [171]; Diabetes [172]; Liver cirhosis and burns [173]; Hepatitis [170,174]; anemia [173]; hemophilia [175]; organophophate poisoning [176]; Hypertension [177] etc. Shenoy et al [28] 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 was made by Biolex therapeutics, regarding Locterin a plant made controlled release interferon alpha treatment for chronic hepatitis [174]. First plant made therapeutic to reach phase III trials was carrot suspension cells derived Gauchers disease therapeutic developed by Prolix BioTherapeutics [178]. 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 UPLYSOTM (alphataliglicerase) 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 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.

Limitations of Plant Vaccines

There may be development of immunotolerance to vaccine peptide or protein. Consistency of dosage form differs to fruit, plant, and generation of the plant. There have been reports of fruit specific expression of vaccines in tomato [179-182] and banana [183] and also in potato tuber [184,185]. Stability of vaccine in fruit is not known. Rigano and Walmsley [186] reported the expression of a fusion protein containing LTB and a species specific immune contraceptive epitope in fresh tomato fruits. As the shelf life of the fruits are less, the fruits were pooled and freeze-dried. Freeze-drying tomato fruit concentrated antigen 16-fold and extended shelf life to 5 months (before materials were used). These materials also proved to be immunogenic in animal trials [180]. Certain plants like potato cannot be eaten raw and cooking may change the properties of vaccine. The efficacy of freeze dried and stored potato based vaccine has also been reported [187]. Evaluating dosage requirement is tedious in case of plant vaccine. Selection of best plant is difficult. There has not been much reports regarding the stability and or characterization of vaccines in fruits.

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 [188]. 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. It is probable that partially purified vaccines would be first introduced for veterinary purposes and then progress to humans.

References

1. Kapila J, De Rycke R, van Montagu M, Angenon G (1997) An agrobacterium-mediated transient gene expression system for intact leaves. Plant Sci 122: 101-108.
2. Gleba Y, Klimyuk V, Marillonnet S (2005) Magnifection--a new platform for expressing recombinant vaccines in plants. Vaccine 23: 2042-2048.
3. Pogue GP, Vojdani F, Palmer KE, Hiatt E, Hume S, et al. (2010) Production of pharmaceutical-grade recombinant aprotinin and a monoclonal antibody product using plant- based transient expression systems. Plant Biotechnol J 8: 638-654.
4. Vézina LP, Faye L, Lerouge P, D'Aoust MA, Marquet-Blouin E, et al. (2009) Transient co-expression for fast and high-yield production of antibodies with human-like N-glycans in plants. Plant Biotechnol J 7: 442-455.
5. Meyers B, Zaltsman A, Lacroix B, Kozlovsky SV, Krichevsky A (2010) Nuclear and plastid genetic engineering of plants: comparison of opportunities and challenges. Biotechnol Adv 28: 747-756.
6. Reddy VS, Sadhu L, Selvapandiyan A, Raman R, Giovanni F, Shukla V, et al. (2002) Analysis of chloroplast transformed tobacco plants with cry1Ia5 under rice psbA transcriptional elements reveal high level expression of Bt toxin without imposing yield penalty and stable inheritance of transplastome. Mol Breed 9: 259-269.
7. Daniell H, Vivekananda J, Nielsen BL, Ye GN, Tewari KK, et al. (1990) Transient foreign gene expression in chloroplasts of cultured tobacco cells after biolistic delivery of chloroplast vectors. Proc Natl Acad Sci U S A 87: 88-92.
8. Daniell H, Datta R, Varma S, Gray S, Lee SB (1998) Containment of herbicide resistance through genetic engineering of the chloroplast genome. Nat Biotechnol 16: 345-348.
9. Daniell H, Lee SB, Panchal T, Wiebe PO (2001) Expression of the native cholera toxin B subunit gene and assembly as functional oligomers in transgenic tobacco chloroplasts. J Mol Biol 311: 1001-1009.
10. Daniell H, Muthukumar B, Lee SB (2001) Marker free transgenic plants: engineering the chloroplast genome without the use of antibiotic selection. Curr Genet 39: 109-116.
11. Daniell H, Khan MS, Allison L (2002) Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology. Trends Plant Sci 7: 84-91.
12. Daniell H, Carmona-Sanchez O, Burns B (2004) Chloroplast derived antibodies, biopharmaceuticals and edible vaccines. In Fischer R, Schillberg S (edr) Molecular Farming. Verlag Publishers, Weinheim, Germany.
13. Daniell H, Chebolu S, Kumar S, Singleton M, Falconer R (2005) Chloroplast-derived vaccine antigens and other therapeutic proteins. Vaccine 23: 1779-1783.
14. Daniell H (2006) Production of biopharmaceuticals and vaccines in plants via the chloroplast genome. Biotechnol J 1: 1071-1079.
15. Daniell H (2007) Transgene containment by maternal inheritance: effective or elusive? Proc Natl Acad Sci U S A 104: 6879-6880.
16. Ma JK, Drake PM, Christou P (2003) The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet 4: 794-805.
17. Ma JK, Barros E, Bock R, Christou P, Dale PJ, et al. (2005) Molecular farming for new drugs and vaccines. Current perspectives on the production of pharmaceuticals in transgenic plants. EMBO Rep 6: 593-599.
18. Ma JK, Chikwamba R, Sparrow P, Fischer R, Mahoney R, et al. (2005) Plant-derived pharmaceuticals--the road forward. Trends Plant Sci 10: 580-585.
19. Koprowski H (2005) Vaccines and sera through plant biotechnology. Vaccine 23: 1757-1763.
20. Lal P, Ramachandran VG, Goyal R, Sharma R (2007) Edible vaccines: current status and future. Indian J Med Microbiol 25: 93-102.
21. Lai H, Chen Q (2012) Bioprocessing of plant-derived virus-like particles of Norwalk virus capsid protein under current Good Manufacture Practice regulations. Plant Cell Rep 31: 573-584.
22. Houdebine LM (2009) Production of pharmaceutical proteins by transgenic animals. Comp Immunol Microbiol Infect Dis 32: 107-121.
23. He J, Lai H, Brock C, Chen Q (2012) A novel system for rapid and cost-effective production of detection and diagnostic reagents of West Nile virus in plants. J Biomed Biotechnol 2012: 106783.
24. Thomas DR, Walmsley AM (2014) Improved expression of recombinant plant-made hEGF. Plant Cell Rep 33: 1801-1814.
25. Hayden CA, Smith EM, Turner DD, Keener TK, Wong JC, et al. (2014) Supercritical fluid extraction provides an enhancement to the immune response for orally-delivered hepatitis B surface antigen. Vaccine 32: 1240-1246.
26. Hayden CA, Fischer ME, Andrews BL, Chilton HC, Turner DD, et al. (2015) Oral delivery of wafers made from HBsAg-expressing maize germ induces long-term immunological systemic and mucosal responses. Vaccine 33: 2881-2886.
27. Shah S, Hayden CA, Fischer ME, Rao AG, Howard JA (2015) Biochemical and biophysical characterization of maize-derived HBsAg for the development of an oral vaccine. Arch Biochem Biophys 588: 41-49.
28. Shenoy V, Kwon KC, Rathinasabapathy A, Lin S, Jin G, et al. (2014) Oral delivery of Angiotensin-converting enzyme 2 and Angiotensin-(1-7) bioencapsulated in plant cells attenuates pulmonary hypertension. Hypertension 64: 1248-1259.
29. Ganapathy M, Perumal A, Mohan C, Palanisamy H, Perumal K (2014) Immunogenicity pf Brugia malayi Abundant Larval transcript-2, a potential filarial vaccine candidate expressed in tobacco. Plant Cell Rep 33: 179-188.
30. Ganapathy M, Chakravarthi M, Charles SJ, Harunipriya P, Jaiganesh S, et al. (2015) Immunodiagnostic Properties of *Wucheraria bancrofti* SXP-1, a Potential Filarial Diagnostic Candidate Expressed in Tobacco Plant, *Nicotiana tabacum*. Appl Biochem Biotechnol 176: 1889-1903.
31. Rybicki EP (2009) Plant-produced vaccines: promise and reality. Drug Discov Today 14: 16-24.

32. Rigano MM, Alvarez ML, Pinkhasov J, Jin Y, Sala F, et al. (2004) Production of a fusion protein consisting of the enterotoxigenic *Escherichia coli* heat-labile toxin B subunit and a tuberculosis antigen in *Arabidopsis thaliana*. Plant Cell Rep 22: 502-508.

33. Kohl TO, Hitzeroth II, Christensen ND, Rybicki EP (2007) Expression of HPV-11 L1 protein in transgenic *Arabidopsis thaliana* and *Nicotiana tabacum*. BMC Biotechnol 7: 56.

34. Kalbina I, Engstrand L, Andersson S, Strid A (2010) Expression of *Helicobacter pylori* TonB protein in transgenic *Arabidopsis thaliana*: toward production of vaccine antigens in plants. Helicobacter 15: 430-437.

35. Dus Santos MJ, Wigdorovitz A, Trono K, Ríos RD, Franzone PM, et al. (2002) A novel methodology to develop a foot and mouth disease virus (FMDV) peptide-based vaccine in transgenic plants. Vaccine 20: 1141-1147.

36. Dus Santos MJ, Carrillo C, Ardila F, Rios RD, Franzone P, et al. (2005) Development of transgenic alfalfa plants containing the Footand- Mouth-Disease Virus structural polyprotein gene P1 and its utilization as an experimental immunogen. Vaccine 23: 1838-1843.

37. Dong JL, Liang BG, Jin YS, Zhang WJ, Wang T (2005) Oral immunization with pBsVP6-transgenic alfalfa protects mice against rotavirus infection. Virology 339: 153-163.

38. Ehsani P, Khabiri A, Domansky NN (1997) Polypeptides of hepatitis B surface antigen produced in transgenic potato. Gene 190: 107-111.

39. Richter LJ, Thanavala Y, Arntzen CJ, Mason HS (2000) Production of hepatitis B surface antigen in transgenic plants for oral immunization. Nat Biotechnol 18: 1167-1171.

40. Kong Q, Richter L, Yang YF, Arntzen CJ, Mason HS, et al. (2001) Oral immunization with hepatitis B surface antigen expressed in transgenic plants. Proc Natl Acad Sci U S A 98: 11539-11544.

41. Joung YH, Youm JW, Jeon JH, Lee BC, Ryu CJ, et al. (2004) Expression of the hepatitis B surface S and preS2 antigens in tubers of *Solanum tuberosum*. Plant Cell Rep 22: 925-930.

42. Nochi T, Takagi H, Yuki Y, Yang L, Masumura T, et al. (2007) Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination. Proc Natl Acad Sci U S A 104: 10986-10991.

43. Oszvald M, Kang TJ, Tomoskozi S, Jenes B, Kim TG, et al. (2008) Expression of cholera toxin B subunit in transgenic rice endosperm. Mol Biotechnol 40: 261-268.

44. Meshcheryakova YA, Eldarov MA, Migunov AI, Stepanova LA, Repco IA, et al. (2009) Cowpea mosaic virus chimeric particles bearing the ectodomain of matrix protein 2 (M2E) of the influenza A virus production and characterization. Appl Mol Bio 43: 685-694.

45. Karaman S, Cunnick J, Wang K (2012) Expression of the cholera toxin B subunit (CT-B) in maize seeds and a combined mucosal treatment against cholera and traveler's diarrhea. Plant Cell Rep 31: 527-537.

46. Marquet-Blouin E, Bouche FB, Steinmetz A, Muller CP (2003) Neutralizing immunogenicity of transgenic carrot (*Daucus carota* L.)-derived measles virus hemagglutinin. Plant Mol Biol 51: 459-469.

47. Rosales-Mendoza S, Soria-Guerra RE, López-Revilla R, Moreno-Fierros L, Alpuche-Solís AG (2008) Ingestion of transgenic carrots expressing the *Escherichia coli* heat-labile enterotoxin B subunit protects mice against cholera toxin challenge. Plant Cell Rep 27: 79-84.

48. Ramírez YJP, Tasciotti E, Gutierrez-Ortega A, Torres AJD, Flores MTO, Giacca M, et al. (2007) Fruit-specific expression of the human immunodeficiency virus type 1 tat gene in tomato plants and its immunogenic potential in mice. Clin Vaccine Immunol 14: 685-962.

49. Sandhu JS, Krasnyanski SF, Domier LL, Korban SS, Osadjan MD, et al. (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.

50. Perea Arango I, Loza Rubio E, Rojas Anaya E, Olivera Flores T, Gonzalez de la Vara L, et al. (2008) Expression of the rabies virus nucleoprotein in plants at high-levels and evaluation of immune responses in mice. Plant Cell Rep 27: 677-685.

51. Soria-Guerra RE, Rosales-Mendoza S, Márquez-Mercado C, López-Revilla R, Castillo- Collazo R, et al. (2007) Transgenic tomatoes express an antigenic polypeptide containing epitopes of the diphtheria, pertussis and tetanus exotoxins, encoded by a synthetic gene. Plant Cell Rep 26: 961-968.

52. Schestibratov KA, Dolgov SV (2005) Transgenic strawberry plants expressing a thaumatin II gene demonstrate enhanced resistance to *Botrytis cinerea*. Scientia Horticulturae 106: 177-189.

53. Qin Y, Teixeira da Silva JA, Zhang L, Zhang S (2008) Transgenic strawberry: state of the art for improved traits. Biotechnol Adv 26: 219-232.

54. Cox KM, Sterling JD, Regan JT, Gasdaska JR, Frantz KK, et al. (2006) Glycan optimization of a human monoclonal antibody in the aquatic plant *Lemna minor*. Nat Biotechnol 24: 1591-1597.

55. Biolex Therapeutics, United States of America.

56. Skarjinskaia M, Karl J, Araujo A, Ruby K, Rabindran S, et al. (2008) Production of recombinant proteins in clonal root cultures using episomal expression vectors. Biotechnol Bioeng 100: 814-819.

57. Martinez C, Petruccelli S, Giulietti AM, Alvarez MA (2005) Expression of the antibody 14D9 in *Nicotiana tabacum* hairy roots. Elec J Biotechnol 8: 170-176.

58. Sunil Kumar GB, Ganapathi TR, Srinivas L, Revathi CJ, Bapat VA (2006) Expression of hepatitis B surface antigen in potato hairy roots. Plant Sci 170: 918-925.

59. Wang Y, Ying C, Qinhua B, Wenbin L, Yongru S, et al. (2001) Using transgenic *Chlorella ellipsoidea* as bio-reactor to produce rabbit defensin (CBA:351835) Europe PMC 11: 1-5.

60. Wang X, Brandsma M, Tremblay R, Maxwell D, Jevnikar AM, et al. (2008) A novel expression platform for the production of diabetes-associated autoantigen human glutamic acid decarboxylase (hGAD65). BMC Biotechnol 8: 87.

61. Tran M, Zhou B, Pettersson PL, Gonzalez MJ, Mayfield SP (2009) Synthesis and assembly of a full-length human monoclonal antibody in algal chloroplasts. Biotechnol Bioeng 104: 663-673.

62. Rasala BA, Muto M, Lee PA, Jager M, Cardoso RM, et al. (2010) Production of therapeutic proteins in algae, analysis of expression of seven human proteins in the chloroplast of *Chlamydomonas reinhardtii*. Plant Biotechnol J 8: 719-733.

63. Smith ML, Keegan ME, Mason HS, Shuler ML (2002) Factors important in the extraction, stability and in vitro assembly of the hepatitis B surface antigen derived from recombinant plant systems. Biotechnol Prog 18: 538-550.

64. Smith ML, Mason HS, Shuler ML (2002) Hepatitis B surface antigen (HBsAg) expression in plant cell culture: Kinetics of antigen accumulation in batch culture and its intracellular form. Biotechnol Bioeng 80: 812-822.

65. Sojikul P, Buehner N, Mason HS (2003) A plant signal peptide-hepatitis B surface antigen fusion protein with enhanced stability and immunogenicity expressed in plant cells. Proc Natl Acad Sci U S A 100: 2209-2214.

66. Kumar GB, Ganapathi TR, Bapat VA (2007) Production of hepatitis B surface antigen in recombinant plant systems: an update. Biotechnol Prog 23: 532-539.

67. Tregoning JS, Clare S, Bowe F, Edwards L, Fairweather N, et al. (2005) Protection against tetanus toxin using a plant-based vaccine. Eur J Immunol 35: 1320-1326.

68. Daniell H, Chebolu S, Kumar S, Singleton M, Falconer R (2005) Chloroplast-derived vaccine antigens and other therapeutic proteins. Vaccine 23: 1779-1783.

69. Daniell H (2006) Production of biopharmaceuticals and vaccines in plants via the chloroplast genome. Biotechnol J 1: 1071-1079.

70. Chen M, Liu X, Wang Z, Song J, Qi Q, et al. (2005) Modification of plant N-glycans processing: the future of producing therapeutic protein by transgenic plants. Med Res Rev 25: 343-360.

71. Li JT, Fei L, Mou ZR, Wei J, Tang Y, et al. (2006) Immunogenicity of a plant-derived edible rotavirus subunit vaccine transformed over fifty generations. Virology 356: 171-178.

72. Shih SM, Doran PM (2009) Foreign protein production using plant cell and organ cultures: Advantages and limitations. Biotechnol Adv 27: 1036-1042.

73. Ramessar K, Capell T, Christou P (2008a) Molecular pharming in cereal crops. Phytochem Rev 7: 759-792.

74. Ramessar K, Capell T, Twyman RM, Quemada H, Christou P (2008) Trace and traceability--a call for regulatory harmony. Nat Biotechnol 26: 975-978.

75. Hood EE, Witcher DR, Maddock S, Meyer T, Baszczynski C, et al. (1997) Commercial production of avidin from transgenic maize: characterization of transformant, production processing extraction and purification. Mol Breed 3: 291-306.

76. Witcher DR, Hood EE, Petersen D, Bailey M, Bond D, et al. (1998) Commercial production of ß-glucuronidase (GUS):a model system for the production of proteins in plants. Mol Breed 4: 301-312.

77. Nochi T, Takagi H, Yuki Y, Yang L, Masumura T, et al. (2007) Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination. Proc Natl Acad Sci U S A 104: 10986-10991.

78. Oakes JL, Bost KL, Piller KJ (2009) Stability of a soybean seed derived vaccine antigen following long term storage, processing and transport in the absence of a cold chain. J Sci Food Agric 89: 2191-2199.

79. Huang AHC (1992) Oil bodies and oleosins in seeds. Ann Rev Plant Physiol Mol Biol 43: 177-200.

80. Parmenter DL, Boothe JG, van Rooijen GJ, Yeung EC, Moloney MM (1995) Production of biologically active hirudin in plant seeds using oleosin partitioning. Plant Mol Biol 29: 1167-1180.

81. Moloney MM, van Roojen GJ, Boothe J (1999) Oil bodies and associated proteins as affinity matrices. United states Patent.

82. Decker EL, Reski R (2007) Moss bioreactors producing improved biopharmaceuticals. Curr Opin Biotechnol 18: 393-398.

83. Decker EL, Reski R (2008) Current achievements in the production of complex biopharmaceuticals with moss bioreactors. Bioprocess Biosyst Eng 31: 3-9.

84. Decker EL, Reski R (2012) Glycoprotein production in moss bioreactors. Plant Cell Rep 31: 453-460.

85. Wang X, Brandsma M, Tremblay R, Maxwell D, Jevnikar AM, et al. (2008) A novel expression platform for the production of diabetes-associated autoantigen human glutamic acid decarboxylase (hGAD65). BMC Biotechnol 8: 87.

86. Dreesen IA, Charpin-El Hamri G, Fussenegger M (2010) Heat-stable oral alga-based vaccine protects mice from *Staphylococcus aureus* infection. J Biotechnol 145: 273-280.

87. Boehm R, Kruse C, Voeste D, Barth S, Schnabl HA (2001) A transient transformation system for duckweed (*Wolffia columbiana*) using Agrobacterium mediated gene transfer. J Appl Bot 75: 107-111.

88. Franklin SE, Mayfield SP (2005) Recent developments in the production of human therapeutic proteins in eukaryotic algae. Expert Opin Biol Ther 5: 225-235.

89. Kim DH, Kim YT, Cho JJ, Bae JH, Hur SB, et al. (2002) Stable integration and functional expression of flounder growth hormone gene in transformed microalga *Chlorella ellipsoidea*. Mar Biotechnolo 4: 63-73.

90. Franklin S, Ngo B, Efuet E, Mayfield SP (2002) Development of a GFP reporter gene for *Chlamydomonas reinhardtii* chloroplast. Plant J 30: 733-744.

91. Surzycki R, Greenham K, Kitayama K, Dibal F, Wagner R, et al. (2009) Factors effecting expression of vaccines in microalgae. Biologicals 37: 133-138.

92. Rasala BA, Muto M, Sullivan J, Mayfield SP (2011) Improved heterologous protein expression in the chloroplast of *Chlamydomonas reinhardtii* through promoter and 5'untranslated region optimization. Plant Biotechnol J 9: 674-683.

93. Specht EA, Mayfield SP (2013) Synthetic oligonucleotide libraries reveal novel regulatory elements in Chlamydomonas chloroplast mRNAs. A C S Synth Biol 2: 34-46.

94. Rasala BA, Mayfield SP (2011) The microalga *Chlamydomonas reinhardtii* as a platform for the production of human protein therapeutics. Bioeng Bugs 2: 50-54.

95. Basaran P, Rodríguez-Cerezo E (2008) Plant molecular farming: opportunities and challenges. Crit Rev Biotechnol 28: 153-172.

96. Paul M, Ma JK (2011) Plant-made pharmaceuticals: leading products and production platforms. Biotechnol Appl Biochem 58: 58-67.

97. Obembe OO, Popoola JO, Leelavathi S, Reddy SV (2011) Advances in plant molecular farming. Biotechnol Adv 29: 210-222.

98. Odell JT, Nagy F, Chua NH (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313: 810-812.

99. Gutiérrez-Ortega A, Sandoval-Montes C, de Olivera-Flores TJ, Santos-Argumedo L, Gómez-Lim MA (2005) Expression of functional interleukin-12 from mouse in transgenic tomato plants. Transgenic Res 14: 877-885.

100. Aziz MA, Sikriwal D, Singh S, Jarugula S, Kumar PA, et al. (2005) Transformation of an edible crop with the pagA gene of *Bacillus anthracis*. FASEB J 19: 1501-1503.

101. 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.

102. Kang TJ, Loc N, Jang M, Yang M (2004c) Modification of cholera toxin B subunit coding sequence to enhance expression in plants. Mol Breed 13: 143-153.

103. Jani D, Meena LS, Rizwan-ul-Haq QM, Singh Y, Sharma AK, et al. (2002) Expression of cholera toxin B subunit in transgenic tomato plants. Transgenic Res 11: 447-454.

104. Li HY, Ramalingam S, Chye ML (2006) Accumulation of recombinant SARS-CoV spike protein in plant cytosol and chloroplasts indicate potential for development of plant-derived oral vaccines. Exp Biol Med (Maywood) 231: 1346-1352.

105. McGarvey PB, Hammond J, Dienelt MM, Hooper DC, Fu ZF, et al. (1995) Expression of the rabies virus glycoprotein in transgenic tomatoes. Biotechnology (N Y) 13: 1484-1487.

106. Satyavathi VV, Prasad V, Khandelwal A, Shaila MS, Sita GL (2003) Expression of hemagglutinin protein of *Rinderpest virus* in transgenic pigeon pea [*Cajanus cajan* (L.) Millsp.] plants. Plant Cell Rep 21: 651-658.

107. Berberich T, Takagi T, Miyazaki A, Otani M, Shimada T, et al. (2005) Production of mouse adiponectin, an anti-diabetic protein, in transgenic sweet potato plants. J Plant Physiol 162: 1169-1176.

108. Drake PM, Chargelegue DM, Vine ND, van Dolleweerd CJ, Obregon P, et al. (2003) Rhizosecretion of a monoclonal antibody protein complex from transgenic toba

109. Hull AK, Criscuolo CJ, Mett V, Groen H, Steeman W, et al. (2005) Human-derived, plant-produced monoclonal antibody for the treatment of anthrax. Vaccine 23: 2082-2086.

110. Morassutti C, De Amicis F, Skerlavaj B, Zanetti M, Marchetti S (2002) Production of a recombinant antimicrobial peptide in transgenic plants using a modified VMA intein expression system. FEBS Lett 519: 141-146.

111. Murray C, Sutherland PW, Phung MM, Lester MT, Marshall RK, et al. (2002) Expression of biotin-binding proteins, avidin and streptavidin, in plant tissues using plant vacuolar targeting sequences. Transgenic Res 11: 199-214.

112. Scheller J, Gührs KH, Grosse F, Conrad U (2001) Production of spider silk proteins in tobacco and potato. Nat Biotechnol 19: 573-577.

113. Zeitlin L, Olmsted SS, Moench TR, Co MS, Martinell BJ, et al. (1998) A humanized monoclonal antibody produced in transgenic plants for immunoprotection of the vagina against genital herpes. Nat Biotechnol 16: 1361-1364.

114. Stoger E, Sack M, Fischer R, Christou P (2002) Plantibodies: applications, advantages and bottlenecks. Curr Opin Biotechnol 13: 161-166.

115. Chen TL, Lin YL, Lee YL, Yang NS, Chan MT (2004) Expression of bioactive human interferon-gamma in transgenic rice cell suspension cultures. Transgenic Res 13: 499-510.

116. Hood EE, Witcher DR, Maddock S, Meyer T, Baszczynski C, et al. (1997) Commercial Production of avidin from transgenic maize: characterization of transformant, Production, processing, extraction and purification. Mol Breed 3: 291-306.

117. Kang TJ, Kim BG, Yang JY, Yang MS (2006) Expression of a synthetic cholera toxin B subunit in tobacco using ubiquitin promoter and bar gene as a selectable marker. Mol Biotechnol 32: 93-100.

118. Kang TJ, Lee WS, Choi EG, Kim JW, Kim BG, et al. (2006) Mass production of somatic embryos expressing *Escherichia coli* heat-labile enterotoxin B subunit in Siberian ginseng. J Biotechnol 121: 124-133.

119. Kumar GB, Ganapathi TR, Revathi CJ, Srinivas L, Bapat VA (2005) Expression of hepatitis B surface antigen in transgenic banana plants. Planta 222: 484-493.

120. Zhong G, Peterson D, Delaney DE, Bailey M, Witcher DR, et al. (1999) Commercial production of aprotinin in transgenic maize seeds. Mol Breed 5: 345-356.

121. Bhat SR, Srinivasan S (2002) Molecular and genetic analyses of transgenic plants: considerations and approaches. Plant Sci 163: 673-681.

122. Butaye KM, Goderis IJ, Wouters PF, Pues JM, Delauré SL, et al. (2004) Stable high-level transgene expression in *Arabidopsis thaliana* using gene silencing mutants and matrix attachment regions. Plant J 39: 440-449.

123. Fischer U, Kuhlmann M, Pecinka A, Schmidt R, Mette MF (2008) Local DNA features affect RNA-directed transcriptional gene silencing and DNA methylation. Plant J 53: 1-10.

124. Spiker S, Thompson WF (1996) Nuclear Matrix Attachment Regions and Transgene Expression in Plants. Plant Physiol 110: 15-21.

125. Streatfield SJ (2007) Approaches to achieve high-level heterologous protein production in plants. Plant Biotechnol J 5: 2-15.

126. Allen GC, Hall GE Jr, Childs LC, Weissinger AK, Spiker S, et al. (1993) Scaffold attachment regions increase reporter gene expression in stably transformed plant cells. Plant Cell 5: 603-613.

127. Allen GC, Hall G Jr, Michalowski S, Newman W, Spiker S, et al. (1996) High-level transgene expression in plant cells: effects of a strong scaffold attachment region from tobacco. Plant Cell 8: 899-913.

128. Laemmli UK, Käs E, Poljak L, Adachi Y (1992) Scaffold-associated regions: cis-acting determinants of chromatin structural loops and functional domains. Curr Opin Genet Dev 2: 275-285.

129. Liu JW, Tabe LM (1998) The influences of two plant nuclear matrix attachment regions (MARs) on gene expression in transgenic plants. Plant Cell Physiol 39: 115-123.

130. Mlynarova L, Jansen RC, Conner AJ, Stiekema WJ, Nap JP (1995) The MAR-mediated reduction in position effect can be uncoupled from copy number-dependent expression in transgenic plants. Plant Cell 7: 599-609.

131. Vain P, Worland B, Kohli A, Snape JW, Christou P, et al. (1999) Matrix attachment regions increase transgene expression levels and stability in transgenic rice plants and their progeny. Plant J 18: 233-242.

132. Gomord V, Faye L (2004) Posttranslational modification of therapeutic proteins in plants. Curr Opin Plant Biol 7: 171-181.

133. Chen M, Liu X, Wang Z, Song J, Qi Q, et al. (2005) Modification of plant N-glycans processing: the future of producing therapeutic protein by transgenic plants. Med Res Rev 25: 343-360.

134. Sturm A, Van Kuik JA, Vliegenthart JF, Chrispeels MJ (1987) Structure, position, and biosynthesis of the high mannose and the complex oligosaccharide side chains of the bean storage protein phaseolin. J Biol Chem 262: 13392-13403.

135. Wilson IB (2002) Glycosylation of proteins in plants and invertebrates. Curr Opin Struct Biol 12: 569-577.

136. Bardor M, Faveeuw C, Fitchette AC, Gilbert D, Galas L, et al. (2003) Immunoreactivity in mammals of two typical plant glyco-epitopes, core alpha(1,3)-fucose and core xylose. Glycobiology 13: 427-434.

137. Cabanes-Macheteau M, Fitchette-Lainé AC, Loutelier-Bourhis C, Lange C, Vine ND, et al. (1999) N-Glycosylation of a mouse IgG expressed in transgenic tobacco plants. Glycobiology 9: 365-372.

138. Hernández M, Cabrera-Ponce JL, Fragoso G, López-Casillas F, Guevara-García A, et al. (2007) A new highly effective anticysticercosis vaccine expressed in transgenic papaya. Vaccine 25: 4252-4260.

139. Major D, Chichester JA, Pathirana RD, Guilfoyle K, Shoji Y, et al. (2015) Intranasal vaccination with a plant-derived H5 HA vaccine protects mice and ferrets against highly pathogenic avian influenza virus challenge. Human Vaccines & Immunotherapeutics 11: 1235-1243.

140. Shoji Y, Farrance CE, Bi H, Shamloul M, Green B, et al. (2009) Immunogenicity of hemagglutinin from A/Bar-headed Goose/Qinghai/1A/05 and A/Anhui/1/05 strains of H5N1 influenza viruses produced in *Nicotiana benthamiana* plants. Vaccine 27: 3467-3470.

141. Spitsin S, Andrianov V, Pogrebnyak N, Smirnov Y, Borisjuk N, et al. (2009) Immunological assessment of plant-derived avian flu H5/HA1 variants. Vaccine 27: 1289-1292.

142. Mortimer E, Maclean JM, Mbewana S, Buys A, Williamson A-L, et al. (2012) Setting up a platform for plant-based influenza virus vaccine production in South Africa BMC Biotechnology 12: 14.

143. Hiatt A, Cafferkey R, Bowdish K (1989) Production of antibodies in transgenic plants. Nature 342: 76-78.

144. De Wilde C, De Rycke R, Beeckman T, De Neve M, Van Montagu M, et al. (1998) Accumulation pattern of IgG antibodies and Fab fragments in transgenic Arabidopsis thaliana plants. Plant Cell Physiol 39: 639-646.

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

146. Kathuria S, Sriraman R, Nath R, Sack M, Pal R, et al. (2002) Efficacy of plant-produced recombinant antibodies against HCG. Hum Reprod 17: 2054-2061.

147. Hein MB, Tang Y, McLeod DA, Janda KD, Hiatt A (1991) Evaluation of immunoglobulins from plant cells. Biotechnol Prog 7: 455-461.

148. Verch T, Yusibov V, Koprowski H (1998) Expression and assembly of a full-length monoclonal antibody in plants using a plant virus vector. J Immunol Methods 220: 69-75.

149. Vaquero C, Sack M, Chandler J, Drossard J, Schuster F, et al. (1999) Transient expression of a tumor-specific single-chain fragment and a chimeric antibody in tobacco leaves. Proc Natl Acad Sci U S A 96: 11128-11133.

150. Rodriguez M, Ramirez NI, Ayala M, Freyre F, Perz L, et al. (2005) Transient expression in tobacco leaves of an aglycosylated recombinant antibody against the epidermal growth factor receptor. Biotechnol Bioeng 89: 188-194.

151. Brodzik R, Glogowska M, Bandurska K, Okulicz M, Deka D, et al. (2006) Plant-derived anti-Lewis Y mAb exhibits biological activities for efficient immunotherapy against human cancer cells. Proc Natl Acad Sci U S A 103: 8804-8809.

152. Villani ME, Morgun B, Brunetti P, Marusic C, Lombardi R, et al. (2009) Plant pharming of a full-sized, tumour-targeting antibody using different expression strategies. Plant Biotechnol J 7: 59-72.

153. Ma JK, Hiatt A, Hein M, Vine ND, Wang F, et al. (1995) Generation and assembly of secretory antibodies in plants. Science 268: 716-719.

154. Sharp JM, Doran PM (2001) Characterization of monoclonal antibody fragments produced by plant cells. Biotechnol Bioeng 73: 338-346.

155. Sharp JM, Doran PM (2001) Strategies for enhancing monoclonal antibody accumulation in plant cell and organ cultures. Biotechnol Prog 17: 979-992.

156. van Engelen FA, Schouten A, Molthoff JW, Roosien J, Salinas J, et al. (1994) Coordinate expression of antibody subunit genes yields high levels of functional antibodies in roots of transgenic tobacco. Plant Mol Biol 26: 1701-1710.

157. Fischer R, Liao YC, Drossard J (1999) Affinity-purification of a TMV-specific recombinant full-size antibody from a transgenic tobacco suspension culture. J Immunol Methods 226: 1-10.

158. Rademacher T, Sack M, Arcalis E, et al (2008) Recombinant antibody 2G12 produced in maize endosperm efficiently neutralizes HIV-1 and contains predominantly single-GlcNAc N-glycans. Plant Biotechnology Journal 6: 189-201.

159. De Muynck B, Navarre C, Boutry M (2010) Production of antibodies in plants: status after twenty years. Plant Biotechnol J 8: 529-563.

160. Giritch A, Marillonnet S, Engler C, van Eldik G, Botterman J, et al. (2006) Rapid high-yield expression of full-size IgG antibodies in plants coinfected with noncompeting viral vectors. Proc Natl Acad Sci U S A 103: 14701-14706.

161. Thomas DR, Penney CA, Majumder A, Walmsley AM (2011) Evolution of plant-made pharmaceuticals. Int J Mol Sci 12: 3220-3236.

162. He J, Lai H, Brock C, Chen Q (2012) A novel system for rapid and cost-effective production of detection and diagnostic reagents of West Nile virus in plants. J Biomed Biotechnol 2012: 106783.

163. Lerouge P, Cabanes-Macheteau M, Rayon C, Fischette-Lainé AC, Gomord V, et al. (1998) N-glycoprotein biosynthesis in plants: recent developments and future trends. Plant Mol Biol 38: 31-48.

164. Garcia-Casado G, Sanchez-Monge R, Chrispeels MJ, Armentia A, Salcedo G, et al. (1996) Role of complex asparagine-linked glycans in the allergenicity of plant glycoproteins. Glycobiology 6: 471-477.

165. Gomord V, Fitchette AC, Menu-Bouaouiche L, Saint-Jore-Dupas C, Plasson C, et al. (2010) Plant-specific glycosylation patterns in the context of therapeutic protein production. Plant Biotechnol J 8: 564-587.

166. Koprivova A, Stemmer C, Altmann F, Hoffmann A, Kopriva S, et al. (2004) Targeted knockouts of Physcomitrella lacking plant-specific immunogenic N-glycans. Plant Biotechnol J 2: 517-523.

167. Cox KM, Sterling JD, Regan JT, Gasdaska JR, Frantz KK, et al. (2006) Glycan optimization of a human monoclonal antibody in the aquatic plant *Lemna minor*. Nat Biotechnol 24: 1591-1597.

168. Vézina LP, Faye L, Lerouge P, D'Aoust MA, Marquet-Blouin E, et al. (2009) Transient co-expression for fast and high-yield production of antibodies with human-like N-glycans in plants. Plant Biotechnol J 7: 442-455.

169. Cramer CL, Boothe JG, Oishi KK (1999) Transgenic plants for therapeutic proteins: linking upstream and downstream strategies. Curr Top Microbiol Immunol 240: 95-118.

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

171. Avesani L, Vitale A, Pedrazzini E, Devirgilio M, Pompa A, et al. (2010) Recombinant human GAD65 accumulates to high levels in transgenic tobacco plants when expressed as an enzymatically inactive mutant. Plant Biotechnol J 8: 862-872.

172. Kusnadi AR, Nikolov ZL, Howard JA (1997) Production of recombinant proteins in transgenic plants: Practical considerations. Biotechnol Bioeng 56: 473-484.

173. de Leede, LG, Humphries JE, Bechet AC, van Hoogdalem EJ, Verrijk R, et al. (2008) Novel controlled-release Lemna-derived IFN-alpha2b (Locteron): Pharmacokinetics, pharmacodynamics, and tolerability in a phase I clinical trial J Interferon Cytokine Res 28: 113-122.

174. Wang X, Su J, Sherman A, Rogers GL, Liao G, et al. (2015) Plant-based oral tolerance to hemophilia therapy employs a complex immune regulatory response including LAP+CD4+ T cells. Blood 125: 2418-2427.

175. Geyer BC, Kannan L, Cherni I, Woods RR, Soreq H, et al. (2010) Transgenic plants as a source for the bioscavenging enzyme, human butyrylcholinesterase. Plant Biotechnol J 8: 873-886.

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

177. Aviezer D, Brill-Almon E, Shaaltiel Y, Hashmueli S, Bartfeld D, et al. (2009) A plant-derived recombinant human glucocerebrosidase enzyme—a preclinical and phase I investigation. PLoS One 4: e4792.

178. Jiang XL, He ZM, Peng ZQ, Qi Y, Chen Q, et al. (2007) Cholera toxin B protein in transgenic tomato fruit induces systemic immune response in mice. Transgenic Res 16: 169-175.

179. Walmsley AM, Alvarez ML, Jin Y, Kirk DD, Lee SM, et al. (2003) Expression of the B subunit of *Escherichia coli* heat-labile enterotoxin as a fusion protein in transgenic tomato. Plant Cell Rep 21: 1020-1026.

180. Ramirez YJ, Tasciotti E, Gutierrez-Ortega A, Donayre Torres AJ, Olivera Flores MT, et al. (2007) Fruit-specific expression of the human immunodeficiency virus type 1 tat gene in tomato plants and its immunogenic potential in mice. Clin Vaccine Immunol 14: 685-692.

181. Loc NH, Long DT, Kim T-G, Yang MS (2014) Expression of *Escherichia coli* Heat-labile Enterotoxin B Subunit in Transgenic Tomato (*Solanum lycopersicum L.*) Fruit Czech J Genet. Plant Breed 50: 26-31.

182. Kumar GB, Ganapathi TR, Revathi CJ, Srinivas L, Bapat VA (2005) Expression of hepatitis B surface antigen in transgenic banana plants. Planta 222: 484-493.

183. Kim TG, Ruprecht R, Langridge WH (2004) Synthesis and assembly of a cholera toxin B subunit SHIV 89.6p Tat fusion protein in transgenic potato. Protein Expr Purif 35: 313-319.

184. Kim TG, Gruber A, Langridge WH (2004) HIV-1 gp120 V3 cholera toxin B subunit fusion gene expression in transgenic potato. Protein Expr Purif 37: 196-202.

185. Rigano MM, Walmsley AM (2005) Expression systems and developments in plant-made vaccines. Immunol Cell Biol 83: 271-277.

186. Matsumura T, Itchoda N, Tsunemitsu H (2002) Production of immunogenic VP6 protein of bovine group A rotavirus in transgenic potato plants. Arch Virol 147: 1263-1270.

187. Penney CA, Thomas DR, Deen SS, Walmsley AM (2011) Plant-made vaccines in support of the Millennium Development Goals. Plant Cell Rep 30: 789-798.

188. Soria-Guerra RE, Rosales-Mendoza S, Moreno Fierros L, López-Revilla R, Alpuche-Solís AG (2011) Oral immunogenicity of tomato derived sDPT polypeptide containing *Corynebacterium diphtheria*, *Bordetella pertussis* and *Clostridium tetani* exortoxin epitopes. Plant Cell Rep 30: 417-424.

189. Paz De la RG, Monroy Garcia A, Mora-Garcia Mde l, Pena CG, Hernandez Montez J, et al. (2009) An HPV 16 L1 based human papilloma virus like particles containing a string of epitopes produced in plants is able to elicit humoral and cytotoxic T cell activity in mice. Virol J 6: 2.

190. Lee SB, Li B, Jin S, Daniell H (2011) Expression and characterization of antimicrobial peptides Retrocyclin -101 and Protegrin -1 in plants to control viral and bacterial infections. Plant Biotechnol J 9: 100-115.

191. Ortogosa SM, Fernandez–San MA, Veramendi J (2010) Stable production of peptide antigens in transgenic tobacco chloroplasts by fusion to the p53 tetramerization domain. Trans Res 19: 703-709.

192. Andrianova E, Krementsugskaia SR, Lugavskaia NN, Mayorova TK, Borisov VV, et al. (2011) Foot and mouth disease virus polyepitope protein produced bacteria and plants induces protective immunity in guinea pigs. Biochemistry (Mosc) 76: 339-346.

193. Meshcheryakova YA, Eldarov MA, Migunov AI, Stepanova LA, Repco IA, et al. (2009) Cowpea mosaic virus chimeric particles bearing the ectodomain of matrix protein 2 (M2E) of the influenza A virus production and characterization. Appl Mol Bio 43: 685-694.

194. Karaman S, Cunnick J, Wang K (2012) Expression of the cholera toxin B subunit (CT-B) in maize seeds and combined mucosal treatment against cholera and traveller's diarrhea. Plant Cell Rep 31: 527-537.