

Plant Platforms for Efficient Heterologous Protein Production

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Abstract Production of recombinant proteins is primarily established in cultures of mammalian, insect and bacterial cells. Concurrently, concept of using plants to produce high-value pharmaceuticals such as vaccines, antibodies, and dietary proteins have received worldwide attention. Newer technologies for plant transformation such as plastid engineering, agroinfiltration, magnification, and deconstructed viral vectors have been used to enhance the protein production in plants along with the inherent advantage of speed, scale, and cost of production in plant systems. Production of therapeutic proteins in plants has now a more pragmatic approach when several plant-produced vaccines and antibodies successfully completed Phase I clinical trials in humans and were further scheduled for regulatory approvals to manufacture clinical grade products on a large scale which are safe, efficacious, and meet the quality standards. The main thrust of this review is to summarize the data accumulated over the last two decades and recent development and achievements of the plant derived

therapeutics. It also attempts to discuss different strategies employed to increase the production so as to make plants more competitive with the established production systems in this industry.

Keywords: biopharmaceuticals, clinical trials, magnification, protein, plantibodies, vaccines, therapeutics

1. Introduction

Protein synthesis is a very complex process, which involves translation of mRNA on ribosomes and post-translational modifications of protein (glycosylation, phosphorylation, and proper folding), required for its stability and precise expression of its biological activity. Being a tightly regulated process, protein synthesis involves sequential activities of enzymes and co-factors at various steps and also linked to other physiological and biochemical factors. Production of a protein outside its natural host system is called heterologous protein production [1,2] and has been extensively reported in yeast and in other microbes [3]. Simultaneously many other non proteinous biomolecules have also been expressed in microbes [4,5]. Heterologous proteins are divided into three major groups: therapeutic proteins (for pharmaceutical use, [6,7]), reagent proteins (for research and study purposes), and industrial proteins (for various industrial applications, [8]). Among these, proteins used as biopharmaceuticals and for therapeutic purposes form an extraordinary class with the stringent quality standards demanding high value [9]. Recently, an *Arabidopsis*-based super-expression system, was developed and used for a structural pilot study of a multi-subunit integral membrane protein complex [10]. Today, the share of biopharmaceuticals is 28.7% of the total global pharmaceutical industry. The opportunities in biopharmaceuticals are growing very rapidly with an

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expected increase from US\$ 200 billion in 2013 to US\$ 500 billion in the year 2020. There are at present approx. 2500 biotech drugs in discovery phase and 1500 biopharmaceuticals undergoing clinical trials [11,12] (<http://www.marketresearchstore.com/report/global-biopharmaceuticals-industry-2015-market-growth-trends-and-45928>).

Bacterial [13], fungal [14,15], plant [16], and animal systems [17,18] have been used since 1970s to produce a variety of recombinant proteins and later explored to produce biopharmaceuticals in addition to industrially relevant proteins. *Escherichia coli* was the pioneer expression system in which first therapeutic protein, somatostatin, was successfully expressed [13]. Insulin was the first FDA approved therapeutic protein produced from *E. coli* expression system [19]. ATryn was first approved as a therapeutic protein produced from transgenic animal (<http://www.transgenics.com/products/atryn.html>). The first examples, reported in 1990, of heterologous protein production using transgenic plant cell suspension culture is a recombinant human serum albumin [16] and chloramphenicol acetyltransferase [20].

However, it is challenging to meet the high-volume demand of heterologous proteins by all those systems because of limitations of cost, cumbersome procedures, and problems in upscale. Production costs are the key issue for recombinant protein production in non-plant sources, and several factors contribute for higher costs. In view of these factors, efficiency of plant cell suspensions cultures of bamboo was employed for the production of exogenous secondary metabolites [21]. Nevertheless, plants offer remarkable platform for large scale production of eukaryotic proteins and are found to be safe, reasonably cheap, and efficacious. The upfront cost of generating recombinant plant material includes standardization of the experimental protocols, cost of growing, harvesting plant material, and the downstream processing and purification cost. Improvement in expression of heterologous protein at cellular and molecular level is one of the practical ways to alleviate these limitations [22]. Goulet *et al.* [23] emphasized the relevance of both considering main stem leaves and axillary stem leaves while modeling heterologous protein production in *Nicotiana benthamiana*. Moreover, they highlighted the potential of exogenously applied growth-promoting hormones to modulate host plant architecture for improvement of protein yields. An ideal expression system should possess (i) capability to produce the required protein with right conformation, (ii) good productivity, (iii) easy handling and maintenance, (iv) safe and economic, (v) affordable downstream processing, and (vi) easy amenability for experimental manipulations.

2. Plants as Expression Systems: Advantages and Disadvantages

Microbial fermentations, animal cultures, or plant systems have been employed for recombinant protein production based on the type of recombinant proteins, demand, and production costs. Each of these production systems are accompanied by their advantages and disadvantages. Animal cell cultures and transgenic animals carrying harmful pathogens or prions increase the downstream processing cost for their separations. Microbial systems even though considered as best systems to obtain large quantities of proteins, they are unable to carry out post-translational modifications involving folding and glycosylation essential for eukaryotic protein activity and efficacy. Further, large scale production of heterologous proteins in microbial system is confined to inclusion bodies and extraction poses hurdles which enhances the downstream cost. Plant cells on the other hand offer advantages over mammalian and microbial systems by producing different types of eukaryotic proteins having precise folding and modification required for its activity. The dual options of using plant platform are, proteins can be produced in field grown plants or in cell cultures to achieve the required yield. Additionally, use of plants ensures enhanced production and inexpensive scale up cost, easy scaling up, high protein quantity, homogeneity, and relative safety. Albeit, there are few shortcomings in use of plants as production platforms such as slow growth cycle, variation in the yield in each cycle, and downstream processing cost. However, some of these limitations can be overcome in some exceptional cases whereby therapeutic proteins are secreted out into the liquid broth or expressed in edible tissues which can be consumed raw. These will ensure in lowering the downstream costs and decrease the yield losses.

Plants are gaining increasing importance as production platforms because of their ability to glycosylate therapeutic proteins which eventually increases the immunogenic potential and receptor binding in addition to resistance to thermal denaturation and protection from proteolytic degradation. Plants are amenable for glyco-engineering wherein efforts are directed mainly on silencing plant-specific N-glycan-processing genes, and/or the introduction of the enzymatic machinery required for synthesis, transport, and transfer of human type sugars onto the recombinant proteins [24–29]. Moreover, Cox *et al.* [30] demonstrated the production of monoclonal antibody without plant specific glycosylation by knocking down the *Lemna minor* endogenous α -1,3-fucosyltransferase and β -1,2-xylosyltransferase genes which resulted in the production of a single major N-glycan species without any

noticeable plant-specific N-glycans. Further, this monoclonal antibody had better antibody-dependent cell-mediated cytotoxicity and effector cell receptor binding activities than the ones expressed in cultured Chinese hamster ovary (CHO) cells.

3. Various Strategies Used for High-level Expression of Heterologous Protein in Plant System

Plants form the main source of food and medicine ever since ancient times and are generally considered to be safe for human use providing a safer alternative host system for production of desired proteins. Plants have been the major intruders on this earth suggesting the production of heterologous protein at larger quantity and at wider host range. In the most recent decade, tremendous progress has been made in heterologous protein production in plants and plant systems have become competitive alternative to the established production technologies that use bacteria, yeast, fungi, insect, and/or cultured mammalian cells. Strategies have been developed and further improved and augmented with increased knowledge and technology to use plant systems for maximum production of heterologous proteins (Fig. 1).

3.1. Stable expression from DNA integrated into the plant genome

Expression of heterologous protein from plant genome involves stable nuclear transformation and integration wherein whole plants can be regenerated, ultimately producing seeds or vegetative tissues which can be maintained under tissue culture conditions or in experimental farms. This involves screening of large number of transgenic lines to select a best line for protein production [31]. However, nuclear transformation ensures posttranslational modifications and subsequent storage or secretion of the proteins depending on the fused signal peptide. The accumulation of protein in cellular compartments, such as ER, chloroplast, mitochondria, and vacuole ensure appropriate folding and assembly resulting in protein stability and bioactivity. Nuclear transformation and expression of proteins have certain merits, but incurs high cost and consumes more time with generally low levels of expression of proteins [32]. The expression levels in nuclear transformation can be increased by utilizing strong promoters, enhancer elements, and augmenting other molecular strategies like codon usage or addition of strong signal sequences. But long production cycles and ability to cross with native plant species has limited its scope for commercialization.

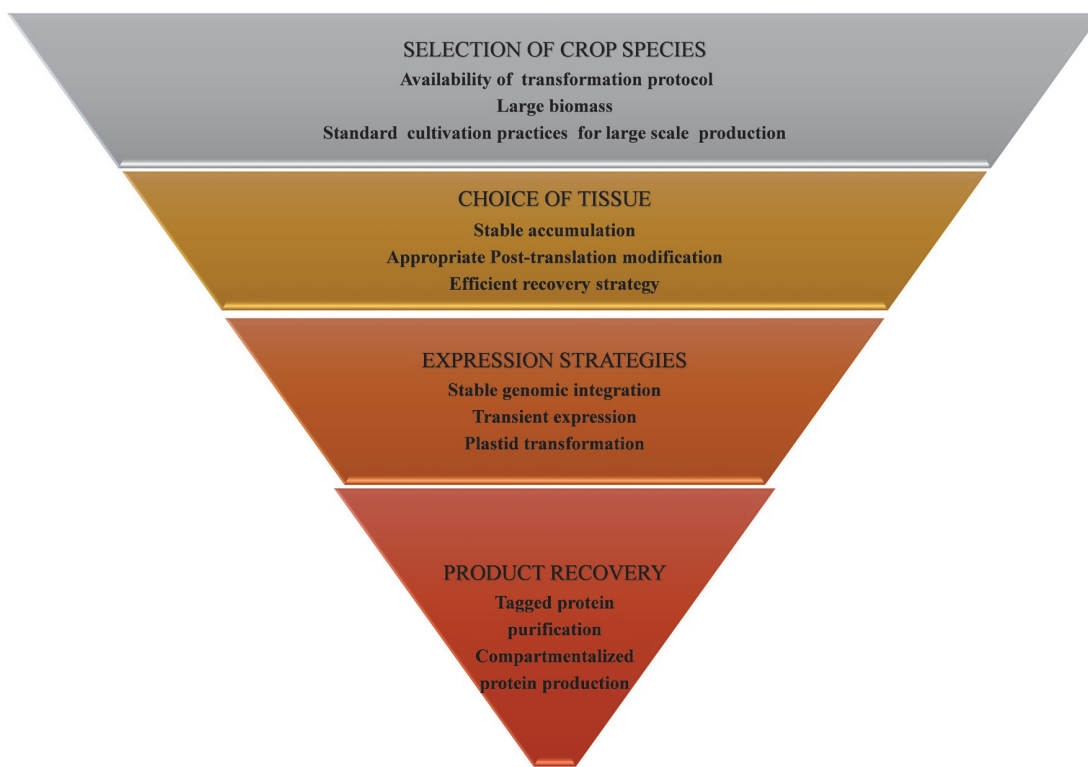


Fig. 1. Approaches for heterologous protein production in plants.

Traditionally, interferon gamma (IFN- γ) which is decisive for immunity against viral, some bacterial and protozoal infections has been produced using a variety of transgenic systems including bacteria, cultured animal cells, and viruses [33-47]. Recombinant IFN- γ expressed in plant and animal systems exhibit different glycosylation profiles, and therefore behaves differently with respect to susceptibility to proteolysis, shorter survival times in blood [48], and high production costs. Chicken interferon gamma (ChIFN- γ) oral vaccine adjuvant was expressed in tobacco plants up to a level of 10 to 20 $\mu\text{g/g}$ fresh leaf weight by applying codon usage and an endoplasmic reticulum retention signal [49]. The heat-labile enterotoxin B (LTB) subunit from enterotoxigenic *E. coli* and the cholera toxin B (CTB) subunit from *Vibrio cholerae* were produced in transgenic rice with a view to produce vaccines against these strains causing diarrhea. LTB and CTB genes were inserted between globulin promoter and potato protease inhibitor II terminator for expression in transgenic rice plants [50].

3.2. Stable expression from the plastid genome (chloroplast transformation / transplastomic technology)

Apart from expression of recombinant protein from plant genome, plastid is also explored as a useful target for genetic manipulation and expression of heterologous proteins. The advantages of chloroplast transformation (transplastomic technology) over the nuclear transformation are: thousands of plastids present in photosynthetic cells of higher plants result in higher level expression (46-70% of the total soluble leaf protein) of transgene with proper folding and formation of disulfide bonds [51-57]. Transgene integrated into chloroplast DNA do not appear to undergo silencing or suffer from position effects due to their site-specific transgene integration into the chloroplast genome; scope for multi-gene engineering in a single transformation event [58,59]; and chloroplast genes are inherited in a strictly maternal fashion providing a natural containment method for transgenic plants, since transgene cannot be transmitted through pollen in majority of plants [60,61].

Integration of the transgene in the transcriptionally active spacer region between the *trnI* and *trnA* genes within the ribosomal operon resulted in the highest levels of transgene expression [62]. Although, chloroplast transformation has been achieved in a few important crop plants such as carrot, potato, tomato, soybean, and eggplant; expressing therapeutic proteins like subunit vaccines in the non-green edible portion is a challenging task. Edible leafy vegetables (lettuce and *Brassica*) could be the suitable options for chloroplast transformation and these could serve as the best source of edible vaccines [63-66].

The chief capsid protein L1 of human papillomavirus HPV-16 was expressed in tobacco chloroplast genome to a

level as high as 240 mg per mature plant. The chloroplast-derived L1 protein exhibited proper conformation and assembled into virus-like particles [67]. Further, intraperitoneal injection of the leaf extract in mice showed presence of neutralizing antibodies [68]. The hepatitis C viral core gene and a codon-optimized gene encoding a C-terminal truncated 16 kDa core polypeptide were expressed in tobacco chloroplasts. Anti-core antibodies in HCV-infected human sera were detected by the 16 kDa core polypeptide in total leaf protein [69]. Youm *et al.* [70] demonstrated successful production of the human b-site APP cleaving enzyme (BACE) in tobacco plants by transplastomic technology. Molecular analysis revealed the integration of tobacco *aadA* and *BACE* genes between *trnI* and *trnA* site in the plastome and transcribed as dicistron. The transgenic tobacco lines accumulated BACE protein at a level of 2% of total soluble proteins and were found to be immunogenic in mice.

3.3. Transient expression

The transient production system is the highest and the most convenient platform for the production of heterologous proteins in plants. The methods employed for transient expression in plants include *Agrobacterium*-mediated transformation or Agroinfiltration [71,72], use of virus based expression system [73,74], and Magniflection technology [75]. Magniflection technology utilizes viral vectors delivered by *Agrobacterium* for high level expression of several polypeptides. Myoglobin, a human heme-protein present in the muscle was successfully produced in the leaves of *N. benthamiana* by transient expression using a viral vector delivered by *Agrobacterium tumefaciens*. The results indicated appropriate incorporation of heme in the protein with comparable functionality as seen in the native system [76].

Among plant-based approaches, viral expression systems have shown great promise and flexibility to produce recombinant proteins on a large scale in short time [77]. Plant virus expression vectors have always been under development as one of the efficient systems for recombinant protein production in plants. Plant RNA virus (*Tobacco mosaic virus*, *Potato virus X*, and *Cowpea mosaic virus*) expression vector systems have been broadly classified as the ones which are engineered for the production of immunogenic peptides and proteins in plants (short epitopes fused to the CP that are displayed on the surface of assembled virus particles) and the other as polypeptide expression systems (expressing the entire recombinant protein) [78]. Viruses are the chosen system for transient expression and transformation as they can be engineered as deconstructed viruses to prevent movement from cell-to-cell, be transmitted by insects to other plants or undergo unnecessary and unexpected recombination events [79].

Fujiki *et al.* [80] developed a *Cucumber mosaic virus* (CMV)-based expression vector for the production of heterologous proteins in plants. The CMV-based expression vector utilized truncated 3a protein, which expresses the target genes from the strong coat protein (CP) sub-genomic promoter and without the need for providing CP *in trans* for cell-to-cell spread. A maximum expression level of ~450 mg/kg and ~170 mg/kg of leaf tissue of green fluorescent protein (GFP) and human growth hormone (hGH), respectively was obtained in *N. benthamiana* plants transformed using agroinfiltration.

Recombinant virus-like particles (VLPs) represent one of the effective vaccine strategies. A stable transgenic plant system for inexpensive production and oral delivery of VLP vaccines have been described previously. On the other hand, the relatively low-level antigen accumulation and long-time frame to produce transgenic plants are the two major obstructions in the practical development of plant-based VLP production. Geminivirus-derived DNA replicon vectors for rapid, high-yield plant-based production of VLPs has been reported by Huang *et al.* [81]. Co-delivery of *bean yellow dwarf virus* (BeYDV)-derived vector and Rep/RepA-supplying vector by agroinfiltration of *N. benthamiana* leaves resulted in efficient replicon amplification and protein production up to 5 days. Co-expression of the P19 protein of *tomato bush stunt virus*, a gene silencing inhibitor, further enhanced VLP accumulation by stabilizing the mRNA. Concurrently, hepatitis B core antigen (HBc) and Norwalk virus capsid protein (NVCP) were produced at a concentration of 0.80 and 0.34 mg/g leaf fresh weight, respectively. This method has advantages of fast and high-level production of VLP-based vaccines using the BeYDV-derived DNA replicon system for transient expression in plants.

Chemical induction using a chemically inducible viral amplicon expression system to increase expression of a heterologous protein, α -1-antitrypsin (AAT), in plants was optimized by Plesha *et al.* [82]. A *cucumber mosaic virus* inducible viral amplicon (CMViva) expression system was used to transiently produce a recombinant human blood protein (AAT), by co-infiltrating intact and detached *N. benthamiana* leaves with two *A. tumefaciens* strains, one containing the CMViva expression cassette carrying the AAT gene and the other containing a binary vector carrying the gene silencing suppressor p19. Application of induction solution every 2 days via topical application resulted in AAT improvement to 1.8-fold of the total soluble protein.

Recent developments in transient expression system involves the combination of plant viral sequences (based on TMV) and *Agrobacterium* binary plasmid (based on pBI121) termed as hybrid Launch vector (pBID4). The

launch vector contains the CaMV35S promoter transcribing the viral sequences. After agroinfiltration multiple single stranded DNA copies present between the left and right borders of the T-DNA of *Agrobacterium* plasmid are produced in the plant cells thereby producing multiple copies of the gene of interest and high expression level. This system has been successfully demonstrated for the expression of influenza H5N1 antigens [83,84].

Magnification is the new strategy employed at the industrial level to assure the requirements like higher-yield, rapid scale-up, and rapid production in bulk quantity. MagnICON® technology (ICON Genetics GmbH, Halle, Germany) is based on *in planta* assembly of functional viral vectors from two separate 5' and 3' pro-vector modules. *Agrobacterium*-mediated transformation helps in delivering these viral sequences into the plant cells which are then assembled in the presence of a site-specific recombinase [75,85]. It was proven highly effective in the production of plague antigens [86], hepatitis B virus core antigen (HBcAg) VLPs [87], Norwalk virus VLPs [88], and anti-ebola monoclonal antibodies [89].

4. Organ Specific Expression

Numerous approaches are employed to express proteins in different organs of plants to achieve improved yield and stability. Foreign proteins can be specifically expressed and/or enriched in leaves, fruits, roots, and seeds of plants (Fig. 2).

4.1. Heterologous protein production in leaves

The idea of producing heterologous protein using plant system gained importance due to its ability to produce large biomass (leaves) under natural conditions. Several tones of biomass can be collected from one to few acres of

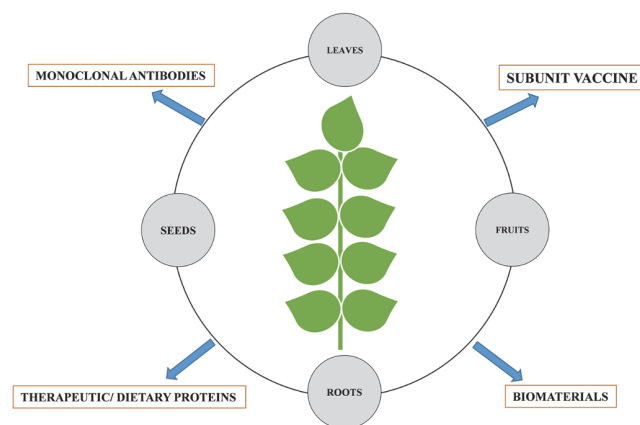


Fig. 2. Schematic representation of heterologous proteins expression in different plant parts.

plantations. Tobacco species namely *Nicotiana tabacum* and *Nicotiana benthamiana* have been the appropriate plant platforms and commonly employed to produce antibodies in leaves. The protocol for stable and transient transformation of tobacco using *Agrobacterium* is very well established. Nevertheless, tobacco plants produce large amount of leaf biomass in a single harvest cycle and also multiple harvest cycles can be performed per year to increase the yield per hectare. Transplastomic technology has provided maximum usage potential of plant leaves for production for therapeutic proteins and vaccines as plastids are capable of producing multimeric proteins with appropriate folds. High yield of active human growth hormone *i.e.*, somatotropin [90] and serum albumin protein [54] have been produced in tobacco chloroplasts. A 47 kDa tetanus toxin fragment (TetC) was successfully expressed in tobacco chloroplasts, and was capable for inducing protective levels of TetC antibodies in mice [91]. Similarly, cholera toxin B fragment fused with human proinsulin CTB-pins [92] and exendin 4 [93] were expressed in high quantities in tobacco leaves and tested for their therapeutic role in treatment of diabetes. *E. coli* enterotoxin B [94] and plague F1-V [95] expressed in tobacco leaves were successful in inducing an immune response and protected mice against these diseases. Nevertheless, several viral antigens were expressed in tobacco leaves with high yield and were effective in eliciting an immune response in mice. Hepatitis E virus HEV E2 [96], Swine fever virus CFSV E2 [97], and human papillomavirus L1 [68] were produced in tobacco chloroplasts. Protozoan antigens such as LecA from *Entamoeba* [98] and CTB-ama1 and CTB-msp1 from *Plasmodium* parasite [99] were produced in tobacco leaves and its potential as vaccine candidates have been evaluated. Besides tobacco, lettuce and alfalfa are the most preferred choice of plants for production of heterologous protein in leaves as they both produce large quantity of leaf biomass and lack harmful phenolic compounds like nicotine. Cholera toxin B subunit fused with the known Mycobacterial antigens; secretory antigens (ESAT6) and cell wall based lipase (LipY) expressed in lettuce chloroplasts were used for oral delivery of TB vaccine antigens [100]. Malarial vaccines namely, Pfama1 and Pfmosp1 were produced in lettuce leaves [99] and were highly immunogenic in mice. Further, the antibodies generated in this response prevented the invasion of *Plasmodium* into red blood cells. A chimeric peptide (MLC) consisting of the merozoite surface protein-1 (MSP-1) and the circumsporozoite protein (CSP) separated by a poly-Gly linker motif expressed in *Brassica napus* was identified as a potential oral vaccine against vivax malaria [101]. Transgenic lettuce expressing the hepatitis B surface antigen (HBsAg) was administered as oral vaccines in mice and humans. This antigen induced HBsAg

antibodies in both populations to a protective level [102]. Geminiviral replicon delivery system was used for high-level expression of virus-like particle (VLP) derived from the Norwalk virus capsid protein (NVCP) and therapeutic humanized mAbs against Ebola (EBV) (6D8) or West Nile (WNV) (hE16) viruses in lettuce [103].

Alfalfa has a strong regenerative capacity in addition to low quantities of phenolic compounds and high content of proteins which makes it suitable for expressing proteins in large quantities. The sVP6 protein of human group A rotavirus expressed in alfalfa elicited sera antibodies in mice and provided passive immunization when subjected to a challenge with simian rotavirus SA-11 [104]. The most successful achievement is the production of animal vaccines in alfalfa. Protective levels of serum antibody were generated to foot and mouth disease virus in mice after oral administration or parenteral immunization with transgenic alfalfa plants expressing the Foot and Mouth disease viral structural protein VP1 [105]. A truncated version of the structural protein E2 from Bovine Viral Diarrhea Virus (BVDV) was expressed as a fused protein in transgenic alfalfa plants [106]. Mice and bovine studies demonstrated production of BVDV specific antibodies that were able to protect the animals completely from infection.

4.2. Heterologous protein production in seeds

Ease of collection, processing, and concentration of protein in seed tissues has made plant seeds as natural bioreactors for successful heterologous protein production. Transgenic plants are engineered to express and accumulate recombinant proteins in seeds by using seed specific regulatory sequences such as tissue-specific soybean seed storage β -conglycinin promoter [107]. Soybeans (*Glycine max*) are the chosen plants for seed specific expression, as these seeds are richest source of proteins and contain nearly 40% protein by dry mass [108]. Transgenic soybean seed derived vaccine was used to protect cattle from infection by enterotoxigenic *E. coli* (ETEC). The ETEC fimbriae subunit protein FanC antigen was overexpressed in soybean and was found to elicit immune response in mice even in the absence of cold chain transportation and storage [109]. Human proinsulin gene was expressed in transgenic soybean seeds driven by monocot tissue-specific promoter from sorghum γ -kafirin seed storage protein gene and the α -coixin cotyledonary vacuolar signal peptide from *Coix lacryma-jobi*. Proinsulin accumulated in soybean seeds and was found to be stable even after 7 years under room temperature conditions [110]. Heat-labile enterotoxin B (LTB) subunit from enterotoxigenic *E. coli* was expressed in soybean seeds [111] and rice seeds [50] and used as oral vaccines to immunize mice against ETEC. The toxin-neutralizing activity of LTB in serum of orally immunized

mice was due to the action of both IgG and IgA responses.

Murine single-chain variable fragment (scFv) was accumulated in transgenic *Arabidopsis* seeds, as high as 12.5% and 36.5% of total soluble proteins using the 5' and 3' regulatory sequences of the seed storage protein gene *arcelin 5-1* and beta-phaseolin seed storage protein promoter from *Phaseolus vulgaris*, respectively [112,113]. Additionally, single-chain variable fragment-Fc antibodies neutralizing hepatitis A virus [114], porcine reproductive and respiratory syndrome virus glycoproteins [115] and human lysosomal acid β -glucosidase protein [116] was expressed in transgenic *Arabidopsis* seeds and assessed for their immunogenicity. Numerous antigens have been expressed in corn seeds owing to high biomass yield, bigger endosperm, absence of active proteases in dry seeds and presence of a rich mix of molecular chaperones and disulfide isomerases for proper folding of proteins and the very well-established processing technology [117,118]. Heat labile B subunit from enterotoxigenic *E. coli*, cholera toxin B subunit from *V. cholerae*, spike protein of swine transmissible gastroenteritis, hepatitis B oral vaccine and human recombinant proinsulin was efficiently expressed in corn plants [119-124].

Rice is another apt candidate next to corn to express heterologous proteins with the additional advantage of self-pollination and cultivated in most parts of the world. Rice seeds were engineered to produce therapeutic proteins like human serum albumin [125], single chain variable fragment against a tumor-associated marker antigen-carcinoembryonic antigen [126], and interleukin 10 [127]. The major T-cell epitopes from Japanese cedar pollen allergens Cryj1 and Cryj2 were expressed in the endosperm of rice seeds [128,129]. Mice fed with these transgenic rice seeds daily for three weeks and then challenged with cedar pollen allergen showed significant suppression of allergen-specific CD4⁺ T-cell proliferation, IgE and IgG levels. Such studies can be further used for developing potential human vaccines for oral administration as tolerogen formulations [130]. Few reports in recent past also demonstrated production of therapeutic proteins in seed tissues such as hepatitis B surface antigen in tobacco seeds [131], hepatitis C core antigen in *B. napus* seeds [132], and anti-hypertensive peptide in rice seeds [133]. Other plants have also been investigated for production of recombinant proteins in seeds but cereals and legumes have been the favored choice. However, there is a strong opposition for producing therapeutic proteins in such food crops because of the contamination issues with non-transgenic plants under non-stringent conditions.

4.3. Heterologous protein production in fruits and vegetables

Fruit can serve as one of the best plant organs for production

of therapeutic proteins and oral delivery of vaccines because they are consumed raw and hence the expressed proteins will retain their natural conformation imparting more immunogenicity. Tomatoes have been used for expression of heterologous proteins owing to the easy availability of transformation protocol, capability to increase biomass with low cost under greenhouse conditions and the short life cycle. Additionally, the amount of protein content in raw tomatoes is more as compared to the ripened tomatoes. *Yersinia pestis* F1-V antigen fusion protein expressed in tomato fruits and administered orally in mice developed immunogenicity when challenged subcutaneously with bacterially produced F1-V [134]. An edible diphtheria-pertussis-tetanus (DPT) multicomponent vaccine was expressed satisfactorily in transgenic tomato plants by combining the exotoxin epitopes from *Corynebacterium diphtheriae*, *Bordetella pertussis*, and *Clostridium tetani* [135].

Proteins expressed in fruit tissue undergo post-translation modification and form biological active oligomers. *E. coli* heat-labile enterotoxin B subunit when expressed in plant was able to form active pentamers and specifically bind to GM1 ganglioside [136]. A range of viral antigenic proteins, such as rabies virus glycoprotein G [137], respiratory syncytial virus F glycoprotein [138], a hepatitis E virus surface protein [139], rotavirus capsid protein VP2 and VP6 [140], a synthetic HBV/HIV antigen [141], Norwalk virus capsid antigen [142], hepatitis B virus surface antigen (HBsAg) [143] chimeric human papilloma virus HPV-16 L1 proteins were expressed in tomatoes [144]. Other industrially important product engineered in tomato fruits includes a taste-modifying protein, miraculin which functions to change the perception of a sour taste to a sweet one [145].

Transgenic banana plants have been used to produce HBsAg in fruits with a view to use it for vaccination through banana fruits [146]. But the lower yield of this antigenic protein in banana fruits raised a problematic technical issue for its use as a vaccine. The difficulty of accumulating heterologous proteins in ripened fruits such as banana has always hindered the system to be used for vaccine delivery. Another study claimed production of cholera toxin B subunit in transgenic banana callus [147].

Strawberries have been used as candidate for production of recombinant proteins as fruits are eaten raw, store good amount of protein and strawberry plants are propagated vegetatively thus lessening the risk of gene contamination. A Japanese firm had setup a plant under the METI project for production of canine interferon (IFN α) from transgenic strawberries [148]. Later these transgenic strawberries expressing dog interferon- α were powdered and sold as an oral drug from 2014 and was found to be effective in the treatment of periodontal disease [149,150].

4.4. Heterologous protein production in roots/tubers

Transgenic potato tubers are used for the last few years as a source for heterologous proteins due to key properties such as long-term storage of accumulated protein in stable form, plentiful of biomass, short growth cycle and certainly the ability to induce mucosal immunity on oral administration [151,152]. Transgenic potato plants constitutively expressing the synthetic *E. coli* heat-labile enterotoxin subunit B (LT-B) gene which has increased accumulation and pentamers assemblage of LT-B in leaves and tubers. Raw tubers fed to mice in three doses were able to generate higher levels of serum and mucosal anti-LT-B antibodies [153]. Cholera toxin B subunit protein was expressed in potato tubers and was able to induce serum and intestinal anti-CTB antibodies [154,155]. Transgenic potato tubers have also been used to produce viral antigens such as hepatitis B surface antigen [156,157], *Norwalk virus* coat protein [158,159], epitope of *porcine epidemic diarrhea virus* [160], GP5 protein of *Porcine reproductive and respiratory syndrome virus* [161], *Rotavirus* VP7 [162], *infectious bronchitis virus* (IBV) S1 glycoprotein [163], and *human papilloma virus* major capsid protein L1 [164,165] or oncogene E7 [166] as oral vaccine candidates. Yu and Langridge [167], constructed a combination of Cholera toxin B and A2 subunit, *rotavirus* enterotoxin and enterotoxigenic *E. coli* fimbrial antigen genes and were expressed in transgenic potatoes. This fusion protein was assembled into cholera holotoxin-like structures having enterocyte-binding affinity and elicited immune response against diarrheal symptoms.

Transgenic potato tubers have also been used to produce other therapeutic proteins and nutraceuticals. Soybean agglutinin (SBA), an N-acetylgalactosamine-binding plant lectin finds application in screening and treatment of breast cancer, fetal cell screening, purification of tagged proteins and carrier for drug delivery. The SBA produced in transgenic potato tubers was highly resistant to degradation by gastric secretions and also retained its specific binding activity [168]. Human beta-casein protein was expressed in potato plants with a view to replace bovine milk in baby foods which is responsible for gastric and intestinal diseases in children [169]. Further, Chong and Langridge [170] also expressed human lactoferrin gene in potato tubers to a level approximately 0.1% of total soluble proteins. A cholera toxin B subunit-Insulin fusion protein was produced in transgenic potato tubers at a concentration of 0.1% of the total soluble proteins. Non-obese diabetic mice fed with these potato tuber tissues showed reduction of inflammation of pancreatic cells and delay in diabetes disease progression [155]. In another study, ricin subunit B from castor bean was used as an immunomodulatory molecule capable of enhancing immunosuppression associated with Type I diabetes [171]. Subtypes of human and salmon interferon

alpha was produced in transgenic potatoes and effectively tested for their bioactivity against viral strains [156-158]. To increase the nutritional quality of potato tubers, *AmA1* gene from *Amaranthus hypochondriacus* was transformed into potato plants in a tuber specific manner. This protein is rich in all essential amino acids for optimal human nutrition [172].

Other plant tubers used for heterologous protein expression includes carrot, sweet potatoes, and turnips. The fusion protein CFP10-ESAT6-dIFN derived from *Mycobacterium tuberculosis* genes was synthesized and used to transform carrot tissue. The protein was produced in transgenic carrot root tissues and was able to induce both humoral and cell-mediated immune responses in mice [173]. *Y. pestis* F1 and V antigens were produced in transgenic carrot roots and were able to elicit protective levels of antibodies [174].

Hairy root cultures have been standardized for the commercial-scale production of secondary metabolites. These root cultures have also been exploited to produce heterologous proteins in larger amounts in continuous bioreactor cultures. Hairy roots offer unique advantages because of their genetic and biosynthetic stability, fast doubling time, require simple hormone free medium and easy scalable protein production. Transgenic tobacco hairy root cultures were engineered to produce human acetylcholinesterase [175], active antimicrobial peptide ranalexin [176], CD20-specific 2B8-FcΔXF recombinant antibody [177], and recombinant human erythropoietin [178]. Tobacco hairy root cultures were also used for the production of murine interleukin 12 [179]. A study demonstrated use of *Brassica rapa* (turnip) hairy root cultures over tobacco cultures for better production capability and stability of heterologous proteins. An isoform of human growth hormone was produced in *Brassica oleracea* hairy root cultures. The *B. rapa* hairy root cultures showed that the recombinant protein is characterized by highly homogeneous post translational profiles enabling a strong batch to batch reproducibility and highlighting the high homogeneity of the production system [180,181]. Although the recombinant proteins are accumulated or secreted by hairy root cultures in large quantities, proteins are degraded by the proteolytic activity of peptidases. Nevertheless, studies are underway to inactivate these peptidases genetically or biochemically.

5. Boosting Plants as Heterologous Protein Production Systems

Research in heterologous protein production in plant is now oriented for increasing the recombinant protein production to cut down overall production and downstream

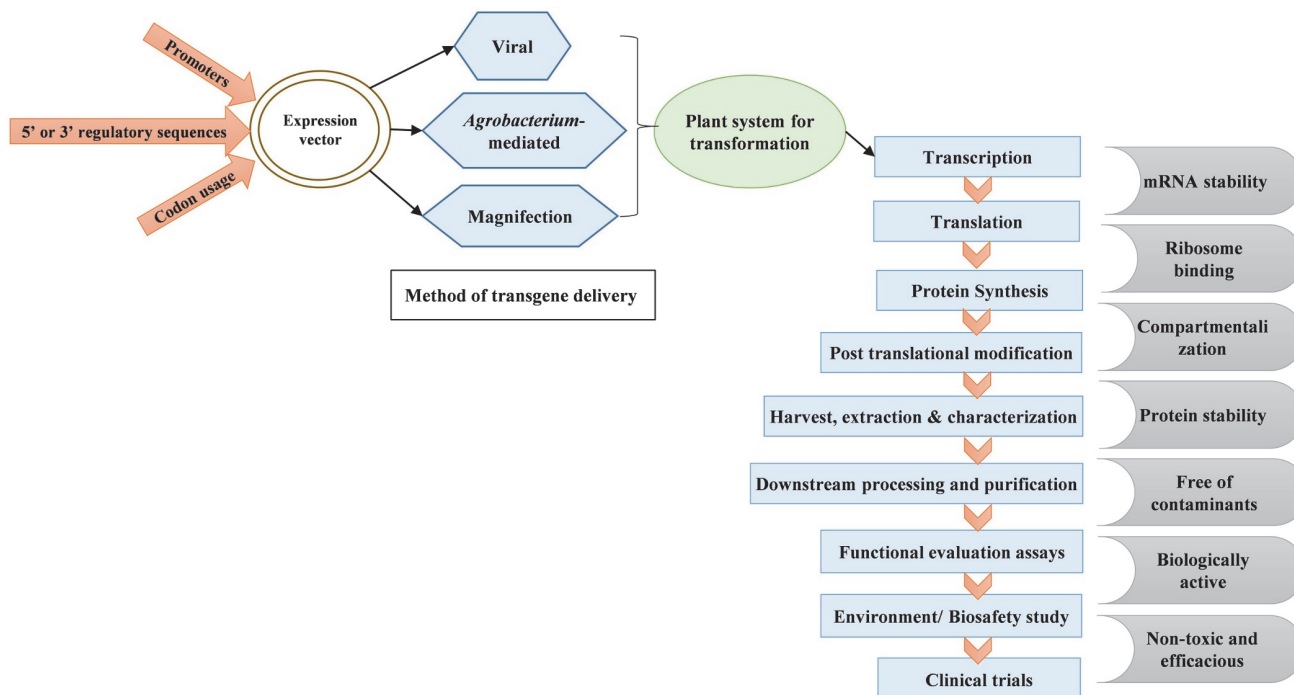


Fig. 3. Schematic representation of stages involved in heterologous protein production in plants along with factors affecting final product output.

cost. Several strategies have been implemented to enhance protein production in plants by modulating regulatory sequences like promoters, 3' UTRs, increasing transcription rate and transcript stability, efficient translation and compartmentalization for higher accumulation (Fig. 3). A number of constitutive and tissue specific promoters have been used for high level protein production. The most widely used constitutive promoter is the *Cauliflower mosaic virus* 35S promoter (CaMV35S) which has shown to drive strong expression of the downstream gene of interest in most of the plant species. Alternatively, other constitutive promoters used for plant transformation include ubiquitin, actin, histone, tobacco cryptic promoter, and nopaline synthase promoter to list a few [182-185]. Chimeric promoters or combination of viral promoters have been created to enhance the expression level. Elements from the *Commelina Yellow Mosaic Virus* (CoYMV), the *Cassava Vein Mosaic Virus* (CsVMV) and activating sequences from the CaMV35S promoter were combined together to drive strong expression of the downstream reporter gene [185,186]. Selected tissue specific promoters have also been utilized to accumulate and compartmentalize recombinant proteins which affect protein stability and downstream processing. Several root, fruit, tuber, and seed specific promoters have been isolated. Furthermore, to achieve high level of expression of the heterologous protein multiple enhancer elements from strong promoters

can be stacked upstream of gene of interest which will increase the transcription rate. Another way to increase the transcriptional activity is to insert scaffold or matrix attachment regions next to the promoter sequence which helps in the recruitment of transcription factors and polymerases. Duan *et al.* [187] heterologously transformed *Arabidopsis* chimeric myosin XI-2 gene in *Camelina* and the transgenic plants exhibited not only enhancement of leaf development, total seed yield and main stem elongation but also early flowering and seed setting, indicating that the high-speed chimeric myosin XI-2 can improve plant growth in *Camelina*.

Inducible promoter systems offer the opportunity to regulate gene expression levels at particular stages of plant growth and development and in particular tissues of interest. Thus, the overexpression of heterologous protein will not hinder normal growth and development and can be accumulated with higher yields at the right stage for easy downstream processing. The expression can be triggered by external elicitors like ethanol, tetracycline, dexamethasone, copper, salicylic acid, salts, and sugars and by environmental factors like temperature and oxidative stress or by pathogens [188]. Dugdale and his team [189] developed a technology termed as INPACT (*In Planta Activation*) used for inducible high level expression of protein in tobacco plants. The INPACT cassette is assembled in such a way that on infection by *tobacco yellow dwarf virus*, the recombinant

gene is split and expressed from the extra chromosomal, replicating episomes which are set free from the host chromosome in the presence of the virus-encoded replication associated proteins, Rep/RepA transcriptionally controlled by the AlcA:AlcR switch responding to ethanol application. Using INPACT technology bovine trypsinogen and human vitronectin was expressed in tobacco leaves to a level of up to 196 mg/kg (dry weight) and ~100 mg/kg (fresh weight), respectively.

Transcription levels mostly cannot be correlated to the amount of protein accumulated as it depends on several factors such as efficient 5'capping, mRNA splicing, polyadenylation, nuclear export, and mRNA stability in the cytosol. However, all these factors can be manipulated to enhance protein production in plant systems. Insertion of a strong 3' UTR downstream to the gene of interest in the expression vector takes care of the polyadenylation and mRNA stability. Commonly used terminators are nopaline synthase, *cauliflower mosaic virus* 35S, heat shock protein and potato proteinase inhibitor II 3'UTRs [190-192]. Position and the sequence of an intron have significant effects on expression levels of the proteins in plant cells. Thus, intron sequences are modulated in the synthetic gene constructs to achieve maximum expression levels [189, 193,194].

Translational efficiency of the protein sequences can be increased by inserting a leader sequence or manipulating native 5'UTR sequence with the objective to increase ribosome binding and thereby translation. In addition to augment protein production, codon optimization can be carried out during designing the gene of interest. During this procedure some rarely utilized codons can be removed and replaced by the codons commonly recognized from the available pool of t-RNAs in plants. Precise engineering is warranted depending upon the type of transformation as codon usage for nuclear and plastid genes are varied. To obtain proteins in their biological active form, they undergo post translational modifications such as glycosylation, phosphorylation, methylation, and ribosylation. Post translational degradation of proteins can be effectively reduced by subcellular compartmentalization. Proteins can be sequestered to organelles like endoplasmic reticulum, vacuoles, mitochondria, and plastids. Addition of ER retention signal (KDEL/ HDEL) or mitochondrial/plastid signal sequences can direct the transport of nascent peptide to the respective organelle. ER targeting helps in safeguarding the proteins and ensures proper folding and assembly because of the resident chaperone machinery [195,196]. C-terminal fusion of the KDEL peptide, an ER retention signal to single-chain antibody variable-region fragments has been found to increase antibody levels by a factor of up to 10-100 as compared to either extracellular

secretion to apoplast or expression in the cytosol [197]. Proteins which can accumulate and are stable under acidic conditions can be sequestered to the vacuoles. Few N-terminal and C-terminal propeptide sequences have been identified that routes the protein to vacuoles. Plastids in plants are the next important organelles to accumulate proteins up to 70% of the total soluble proteins. Proteins can be diverted to plastids by adding unique signal sequences or can be inherently produced in plastids by transplastomic technology described before. Most proteins produced and channelized through endomembrane system find their way to the apoplast. The proteins secreted into the apoplastic region avert intracellular and vacuolar proteinases. If these proteins are large enough, they accumulate between the cell membrane and cell wall and if small then are secreted into the medium. Secretion of proteins into the medium reduces the downstream cost and helps in easy recovery of the proteins. However, these secreted proteins are constantly exposed to harsh conditions or some extracellular proteinases which degrade the proteins and reduce the production yield. Co-expression of proteinase inhibitors or protease-resistant fusion constructs have increased the production of recombinant proteins in plant systems [198-202].

6. Bioreactors for Recombinant Protein Production in Plant Cells

Plant cell suspension cultures offer best platform for stable accumulation and secretion of recombinant protein in large quantities. Generally, plant nutrient media are relatively simple salt solutions with no added proteins and if a heterologous protein is produced in tissue culture and secreted into the medium, product recovery and purification could be easily and efficiently worked out due to absence of large intracellular contaminating proteins. Several studies have demonstrated secretion of proteins into medium which are less than 30 KDa whereas higher molecular weight ones are retained inside the cell due to plant cell wall. This problem is overcome by incorporating a signal sequence before the gene of interest so as to secrete the protein into the culture medium. Use of bioreactors have facilitated up scaling and increase the production of recombinant proteins to a level equivalent to bacterial or mammalian system [203,204]. Although, the technology of producing recombinant proteins in cell suspension cultures was proven more than 25 years before, the cost and potential to scale up to industrial level was not a very successful venture [16]. During this time, different bioreactors were designed to overcome this hurdle that included the stirred tank and wave bioreactor. Both stirred tank and

airlift bioreactor were used for the production of murine granulocyte macrophage-colony stimulating factor (mGM-CSF) in transgenic *N. tabacum* cells [205]. A new type of bioreactor designed by Protalix Biotherapeutics consisted of large polyethylene bags serially arranged and filled with medium and supplied with sterile air to produce recombinant human β -glucocerebrosidase [206].

Carrot cells and tobacco BY-2 cells are commonly used cell cultures for production of therapeutic, pharmaceutical, and vaccine proteins [206,207]. A number of therapeutic proteins have also been expressed in rice cell cultures. Following are a few proof of principle studies demonstrating the production of foreign protein in rice cell suspension cultures. Human cytotoxic T-lymphocyte antigen 4-immunoglobulin (hCTLA4I g) driven by rice alpha-amylase 3D (RAmy3D) promoter was expressed in transgenic rice cell suspension culture. Purified hCTLA4IgP was found to be biologically active and suppressed T-cell proliferation [208]. mGM-CSF fused with the α Amy3 signal peptide was produced in rice cell suspension cultures scaled up in a 2-L bioreactor and yielded highest concentration of 24.6 mg/L protein in the culture filtrate [209]. A Der p2-FIF-Fve fusion protein was expressed in rice cells under the control of sucrose starvation induced α -amylase gene (α Amy8) the fusion protein elicited IgE immunogenicity and histamine release [210]. Similarly, recombinant alpha-1-antitrypsin [211], human growth hormone [212], and Interferon-gamma (IFN- γ) [44] was also expressed in rice cells.

Currently mosses such as *Physcomitrella patens*, have been employed as platforms for production of therapeutic proteins in bioreactors [213,214]. Owing to their photosynthetic property and glycosylation capability, *P. patens* has become a best alternative to produce high value humanized proteins with reduced production costs. Simple designed bioreactors are employed to grow this moss for production of therapeutic proteins such as vascular endothelial growth factor [215], erythropoietin [216], the complement-regulatory protein factor H [217], HIV epitopes from gp120 and gp41 [218], glyco-optimized antibody IgG1 IGN314 [219], and enzymes such as human alpha-galactosidase and glucocerebrosidase [220]. With increasing knowledge and technical know-how plant cell cultures can be efficiently utilized to produce large quantities of therapeutic proteins at par with bacterial and mammalian platforms with reduced cost.

7. Plant Derived Protein in Clinical Trials

7.1. Antibodies

To date, several antibodies have been synthesized in a

variety of plants having applications in medical, industrial, and research fields. Production of antibodies in plants has gained importance owing to its reduced cost. Effective protection against some serious diseases like AIDS requires continuous application of HIV neutralizing antibodies [221,222] which will dramatically increase the cost per application. In such cases, scaling up production becomes more important than speeding the reaction; and therefore transgenic plant production platform become more pertinent because of their rapid scaling up capacity. In one prominent study antibody cocktails (ZMapp) produced in tobacco plants (*N. benthamiana*) using MagnICON technology was used for treating Ebola infection in non-human primates [89]. Off lately, a high level production of monoclonal antibodies using an optimized plant expression system has been reported [223].

A few number of antibodies expressed in plants reached the clinical trials and soon to be launched in the market. A chimeric secretory antibody CaroRx (IgG-IgA) that binds to the bacteria *Streptococcus mutans*, a causative agent of tooth decay, was expressed in tobacco plants. This antibody is effective as it protects against dental caries and prevents the recolonization of the bacteria up to 2 years after 3 weeks of application [224]. An anti-CD20 optimized antibody BLX-301 was produced in aquatic plant *L. minor* (duckweed) by Biolex Inc. which entered the phase I trials for the treatment of non-Hodgkin's B cell lymphoma and rheumatoid arthritis [30]. MAPP66 is an antibody cocktail produced in *N. benthamiana* by Icon Genetics (Bayer's) MagnIcon technology and used as a HSV/ HIV microbicide which entered first phase clinical trial (Mapp Biopharmaceutical, Inc.). Four anti-HIV neutralizing monoclonal antibodies namely b12, 2G12, 2F5, 4E10 were found to be effective in controlling the transmission of virus [225]. Out of these four antibodies, 2G12 antibody produced in transgenic tobacco by Pharma-Planta has been approved for the first-in human phase I clinical trial in UK [226]. The success of these trials will mark a significant achievement in the field of plant derived pharmaceuticals and will further boost in the transfer of proof of principle studies to commercialization.

Few plant derived antibodies have also been used in manufacturing other biopharmaceuticals. CIGB, a Cuban company, has developed and produced ScFv monoclonal antibody (CB-Hep1) in transgenic tobacco which has been used for several years for the purification of recombinant Hepatitis B subunit vaccine [227].

7.2. Antigen/subunit vaccines

Plant based recombinant vaccines can be categorized as one used for veterinary and other as human vaccines. Plant-based human vaccines are not yet commercialized,

although, proof-of-principle studies of viral and bacterial subunit vaccines have been successfully demonstrated in transgenic plants such as tomato, potato, banana, maize, alfalfa, and soybean. Developing countries are most of the time inflicted with diseases that results in mortality. Plants serve as the best alternative for production of vaccine candidates with reduced cost and thereby decrease the overall cost burden involved in conventional methods for procuring recombinant vaccines. The first plant derived vaccines approved for clinical trial was for the veterinary use which protect against Newcastle disease [228]. Dow AgroSciences (USA) produced USDA approved hemagglutinin and neuraminidase of Newcastle disease virus in tobacco cells for subcutaneous application, but is yet to be marketed [78]. Only few of the plant derived human vaccine subunits reached the clinical trials. Heat-labile enterotoxin B subunit (LTB) of enterotoxigenic *E. coli* (ETEC) produced either in potato or maize was the first to enter clinical trial to protect against diarrhea. Raw mashed transgenic potato or corn meal suspended in water and administered orally to healthy volunteers was observed for its safety and immunogenic potential. The study showed that volunteers fed with transgenic tissues had increased levels of LTB-specific serum IgG and IgA as compared to the placebo controls who were fed with non-transgenic potato/corn [229,230]. In another study, a major capsid protein of *Norwalk virus* was expressed in transgenic potato and used to feed 24 healthy volunteers [159]. Each volunteer was administered 2-3 doses as raw diced potatoes containing 215-751 mg of NVCP. Ninety-five percent of the volunteers showed significant levels of serum IgG and stool IgA. This vaccine is currently being optimized for commercialization under the trade name NoroVAXX [231].

Hepatitis B virus disease persists even after vaccines were developed more than three decades ago. Despite of several attempts to develop oral vaccines for Hepatitis B viral disease in plants like potato and banana, no plant derived vaccine could be commercialized so far but expected to yield promising results in the near future. However, purified antigens derived from plants were immunogenic, but the inherent levels of these antigens in the tissues were found to be considerably low for using it as an edible vaccine. Nevertheless, HBsAg expressed in transgenic potato and lettuce plant was used as oral vaccines for phase I trials. More than 50% of the volunteers fed with transgenic potato showed increased levels of anti-HBsAg antibodies in the serum and developed systemic resistance response to Hepatitis B infection [151,156]. Likewise, HBsAg expressed in transgenic lettuce leaves (0.1-0.5 µg of HBsAg per 100 g of fresh tissue) were given to adult volunteers with reducing doses in two consecutive months [102]. Two of three vaccinated volunteers showed presence

of HBsAg-specific IgG, 2 weeks after the second vaccination. Further, no IgA specific antibodies for HBsAg were detected and there were no noticeable side effects observed after ingestion of transgenic lettuce.

Endemic rabies is the common cause of mortality in some parts of the world. Therefore, there is a need for constant supply of rabies vaccines in these regions. Two rabies virus epitopes, glycoprotein (GP) and nucleoprotein (NP) were fused together and expressed in transgenic spinach under the driving control of recombinant *Alfalfa mosaic virus* machinery [232-234]. Raw spinach leaves were fed to two groups of volunteers, one group who were previously vaccinated for rabies virus and the other non-vaccinated group. In all more than 50% of the volunteers from both the groups displayed elevated levels of sera IgG able to neutralize rabies virus particles.

Influenza virus is a frequently mutating strain that results in the antigenic shift which quite often obliterates cross-protective immunity of the host. In such a scenario, strain specific vaccines produced on a large scale in short period of time are the prime requirement to prevent disease pandemics. D'Aoust and team [235] expressed haemagglutinin (HA) from strains A/Indonesia/5/05 (H5N1) and A/New Caledonia/20/99 (H1N1) by agroinfiltration in *N. benthamiana* plants. The virus-like particles assembled and accumulated in the apoplastic region of tobacco cells and was able to elicit immune response in mice. Phase I / II clinical trial of the VLP composed of HA protein of H5N1 influenza virus (A/Indonesia/5/05) (H5-VLP) has been completed. Both H1 and H5 VLP vaccines elicited significantly greater CD4⁺ T cell responses than placebo and persisted even after 6 months of vaccination [236,237]. Additionally, some volunteers developed antibody response to plant glycans which subsided within 6 months in most volunteers [238]. Medicago Inc. have taken up this project and completed phase I trial with the H1N1 epitope and further the test for trivalent synthetic vaccine for other strains of influenza is underway.

Few years back the rapid spread of H1N1 strain in the developing countries urged the need to produce cheap vaccines in a large quantity. Plant based VLPs were produced to develop subunit vaccines which are immunogenic. Recombinant hemagglutinin proteins from A/California/04/09 (H1N1) and A/Indonesia/05/05 (H5N1) strains of influenza virus were produced in *N. benthamiana* plant on a large scale. The production of serum hemagglutination inhibition and virus neutralizing antibodies was studied in laboratory animals [239]. To further enhance the overall potency of these antigens as vaccine candidates, Shoji *et al.* [240] constructed H1 HA VLPs (HAC-VLPs) using ectodomain of HA from A/California/04/09 strain. The recombinant hemagglutinin protein (HAI-05) from the A/

Indonesia/05/2005 (H5N1) strain of influenza virus was produced transiently in *N. benthamiana* using 'launch vector'. In the phase I clinical trial, the immune response elicited in volunteers by the HAI-05 vaccine was variable with respect to both hemagglutination-inhibition and virus neutralization antibody response [241]. Correspondingly, a first-in-human, Phase I dose dependent study was conducted to investigate safety, reactogenicity, and immunogenicity of an HAC1 formulation at three dosages with and without Allhydrogel (®), in healthy adults 18-50 years of age [242]. In both the phase I trials, the vaccine was generally safe and was well tolerated, with no reported serious adverse events.

Fraunhofer Center for Molecular Biotechnology, Plymouth, MI, USA completed phase I trials for recombinant protective antigen (rPA) against anthrax disease in the year 2014. *Plasmodium falciparum* surface protein Pfs25 expressed in *N. benthamiana* plants by tobacco mosaic virus-based launch vector was able to induce serum antibodies with complete transmission blocking activity [243]. The Pfs25-VLP is in phase I trial and currently undergoing optimization (Fraunhofer Center for Molecular Biotechnology, USA). All these studies demonstrate the safety and immunogenicity of a plant-produced subunit vaccine in healthy adults and propose testing of novel candidate vaccines in human volunteers and further commercialization of these plant derived vaccines to combat severe diseases.

Presently with novel coronavirus responsible for an ongoing human pandemic (COVID-19), researchers have offered plants as platform for rapid supply of SARS-CoV-2 antigens and antibodies for diagnostic, vaccines, and treatment therapies. In a proof-of-concept study the receptor binding domain (RBD) of SARS-CoV-2 was transiently expressed in *N. benthamiana* to a level of 8 µg/g leaf fresh weight at 3 days post-infiltration [244]. Biopharmaceutical companies such as ZYUS Life Sciences Inc. and iBio, Inc. are using plant-based expression system for producing SARS-CoV-2 antigens.

7.3. Therapeutic proteins/nutraceuticals

Several recent studies have validated the potential of nutraceuticals to improve health and prevent chronic illnesses like cardiovascular, cancer, diabetes, obesity, and multiple immune dysfunctions. Because of its nutritional benefits, safety, and therapeutic role, the market for nutraceuticals is expanding every year. Plant derived products such as flavonoids, sterols, tannins, and glucosinolates are the important source of such nutraceuticals and increasing the amount of these metabolites is the primary goal for commercialization. Recombinant glucocerebrosidase called as Elelyso (taliglucerase alfa) was produced in carrot cells for the treatment of Gaucher disease [245]. Gaucher's

disease is a lysosomal storage disorder caused by mutations in the gene encoding glucocerebrosidase (GCD) resulting in the deposition of lipids in spleen, liver, and other organs. Taliglucerase alfa produced by Protalix Biotherapeutics (Israel) and licensed by Pfizer (USA) was administered orally into human patients. Phase I clinical trials displayed presence of the enzyme in the blood stream of the patients with no side effects. Phase 2a, 2b, and 3 trials are under way along with other pharmacokinetics studies.

SemBioSys Genetics Inc., a Canadian biotechnology company produced an insulin molecule in safflower (*Carthamus tinctorius*) at commercially viable levels [246]. The Phase I/II clinical trial conducted in Europe has demonstrated clinically significant results and safety profile comparable to pharmaceutical grade human insulin. The Company submitted the Investigational New Drug application to the US Food and Drug Administration and prepared for first clinical trial in 2008 and planned phase 3 trials in 2009-10.

Recombinant gastric lipase for the treatment of pancreatic and cystic fibrosis was produced in maize seeds and entered phase II clinical trials in Europe in the year 2004 and currently marketed under the brand name Meripase (<http://www.meristem-therapeutics.com>). Maize modified with human lactoferrin (Lacromin™) was field tested by Biochem SA Company and by Meristem Therapeutics Company in France for the treatment of gastrointestinal infections [247]. Ventria Biosciences obtained approvals to carry out field trials (later disapproved by US Food and Drug Administration) of transgenic rice expressing lactoferrin and lysozyme intended for production of iron supplements and antimicrobial activity [248-250]. Recombinant Human Intrinsic Factor useful for vitamin B12 absorption was engineered in *Arabidopsis* seeds and is marketed by Cobento Biotech AS [251].

Field trials of low-nicotine tobacco varieties expressing human interleukin-10 (HIL-10) was carried out in Canada (Southern Crop Protection and Food Research Centre, Canada). HIL-10 which is used for the treatment of inflammatory bowel syndrome and Crohn's disease was produced in tobacco. Oral administrations of transgenic tobacco expressing HIL-10 reduce the severity of colitis by down-regulating TNF- α expression in IBD-susceptible IL-10(-/-) mice [252]. Biolex (USA) used duckweed plants to produce fibrinolytic drug for blood clots and lactoferrin for Hepatitis B & C virus disease which are under phase I and phase II trials respectively (Biolex Therapeutics Inc.). Planet Biotechnology's (<http://www.planetbiotechnology.com>) α -galactosidase (for Fabry disease) produced in tobacco and SemBioSys's (<http://www.sembiosys.com>) Apolipoprotein (for cardiovascular problems) produced in safflower were also approved for phase I trials and expected to reach

market soon. Few of the therapeutic proteins such as virtonectin (Farmacule Bioindustries Pty Ltd) and thyroid stimulating hormone receptor (NEXGEN Biotechnologies, Inc.) are used mostly for research purposes and available from the company. Epidermal growth factor produced in tobacco plants are supplied by Plantderma (<http://plantaderma.es/es/>), used in cosmetology for improving skin properties.

8. Concluding Remarks

Production of antibodies, vaccines, and other therapeutic proteins in plants shows great promise as recombinant proteins can be rapidly produced on large scale with low cost compared to other production systems. Most of the plant derived pharmaceuticals are in clinical trials and many are under investigation. Governing bodies in developed countries are involved in addressing and conceptualizing the manufacturing and application guidelines to ensure safety, efficacy, and consistency of these plant derived pharmaceuticals. Over the last few decades biofarming in plants has made significant progress to retort several shortcomings in the production system and regulatory issues (biosafety and risk assessment) and reached a stage where it can challenge the established production technologies that use bacteria, yeast, and mammalian cells. With the successful application of plant derived ebola vaccine ZMapp by Mapp Biopharmaceutical Inc., to combat the 2014 Ebola virus outbreak in Africa has once again evoked the interest of plant derived pharmaceuticals. The plant biotechnology community is extremely energetic in providing plant-based therapeutics to tackle the current COVID-19 pandemic. This will create a prototype for producing low cost, rapid, and effective therapeutics in plants for future pandemics. Current scenarios have changed the mindset from using plants as edible vaccines to utilizing them as production platforms. Additionally, proteins produced in plants are properly folded and post-translationally modified which can further be formulated as vaccines and therapeutics. Bioreactor is the most promising approach to effectively use plant cells to produce heterologous proteins which involves low capital investment with enhanced product yield. Multinational companies like Protalix, Bayer, Icon Genetics, Meristem therapeutics are testimony to this. The studies carried out at several institutes and industries all over the world with a view to commercialize these products explain the tremendous potential of protein production in plants. Although the scientific community is aware of this fact, still an extensive work is warranted to establish plant production platform as unanimously accepted approach for vaccine and therapeutic

protein production. Remarkable progress in molecular biology currently underway across the globe will definitely open new feasible options for the production of several bio pharmaceuticals. Additionally, synthetic biology is an enabling machinery that aims to increase extrapolative capacity and reconcile involvedness in living systems and may be defined simply as the engineering of biology; driven by technological advances in DNA synthesis, advances in computational methods, and the elucidation of complex heterologous synthesis of biomolecules [253]. Considering this, heterologous protein production in plants would open up for new technologies for developing an efficient and resourceful platform similar to the work on non proteinaceous biomolecules [254]. In this review, we highlighted the past attempts and recent progress in heterologous protein production in plants and its potential for commercial drug development and production. In conclusion, although plants as production platform face problems for public acceptance, undoubtedly it will definitely find its way into future as the best production platforms with constant support from the government, medical field, companies, and scientific community.

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Conflict of Interest

Authors declare that no conflict of interest exists.

Ethical Statement

Neither ethical approval nor informed consent was required for this study.

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