Use plant based-recombinant proteins in food and human health

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Recombinant protein is a manipulated form of protein, which is generated in various ways to produce large quantities of proteins, modify gene sequences and manufacture useful commercial products. The formation of recombinant protein is carried out in specialized vehicles known as vectors.

Plants are chosen because they are ideally suitable for producing recombinant proteins with many advantages over other systems. First, compared to the traditional microbial fermentation, plants are able to perform post-translational modifications, such as glycosylation and hydroxylation, which are required for biological activity of numerous mammalian proteins. Second, plants, especially crops, can produce exogenous proteins on large scale at very low cost, since plant growth, transport, and post-harvest process requirements are relatively inexpensive . Third, unlike animal or microorganism systems, plant-derived products reduce contamination risk due to human pathogens. Fourth they are easily transformed and provide a cheap source of protein. There are also drawbacks for transgenic plant production; one of the concerns is that the timescale of production is relatively long compared with plant or animal cell cultures and microorganisms

Most major groups of proteins have been produced successfully in a diverse variety of crops such as maize, rice, wheat, soybean, tomato, potato, mustard, oilseed rape, turnip, alfalfa, banana and tobacco. We will briefly review the recent developments in plant-based recombinant production technology.

Keywords: recombinant proteins; plant-derived products; protein crops

1. Plant made recombinant proteins

As a result of fast developing biotechnology during past few decades, nowadays plants can be used to produce various heterologous proteins, including pharmaceutical and industrial proteins, through recombinant DNA technology, often referred to as plant molecular farming [1]. Plant-based systems provide the spectrum of production capacity ranging from plant/algal cell bioreactor systems for lower volume, higher value product to field-grown commodity crops with potential for metric tons of recombinant protein at highly competitive costs. Because plants cannot harbor the human and animal pathogens-of-issue for mammalian cell-based production systems, they bring significant advantage in increased safety for patients[2].

Plant-made recombinant proteins can be generally categorized into three classes: therapeutic proteins, industrial proteins/enzymes and biopolymers

1.1 Therapeutic proteins

Therapeutic proteins include monoclonal antibodies (mAbs), vaccine antigens, therapeutic enzymes, blood proteins, cytokines, growth factors and growth hormones[2]. Bioactivity of these proteins often requires protein folding, disulfide bond formation, subunit assembly, proteolytic cleavage, and/or glycosylation, highlighting the ability of plants to process complex human/mammalian proteins. Plant-made antibodies have received considerable interest as they are made at much lower cost in plants than inmammalian cells without the associated risks of potentially harboring animal pathogens [3]. Plants successfully glycosylate proteins at the signature recognition motif (N-X-S/T) for N-linked glycosylation. However, subsequent processing in the Golgi to complex glycans differs from that found in mammalian cells. Thus, a notable challenge in using plants as hosts for production of glycosylated therapeutic proteins is that plantspecific xylose and α-1,3-fucose sugars may be added with a potential to alter bioactivity or immunogenicity in humans[4]

Antibodies and vaccines are the two major classes of plant-made therapeutic proteins that are under commercial development. Planet Biotechnology took the lead in developing and commercializing Plantibodies produced in tobacco; those being tested in clinical trials include CaroRX (for dental caries), DoxoRX (for side-effects of cancer therapy), RhinoRX (for Rhinovirus prophylactic) and an IgG (ICAM1) (for common cold) [5] In addition, a 2G12 IgG used as prophylactic treatment for HIV is in Phase I clinical trials by the Pharma-Planta Consortium) [6], and a plant-made scFv monoclonal antibody, used in downstream processing of a hepatitis B vaccine, has been commercialized in Cuba) [7].

1.2 Industrial enzymes

Transgenic plants provide a viable technology for producing industrial proteins, in particular enzymes, because of low cost of agricultural production, stability of protein stored in specific organs such as seeds, ease and speed of scale-up as

well as the possibility of using crude plant materials directly in industrial processes [8]. These are hydrolases, including glycosidases (e.g., cellulase, α-amylase and β-glucuronidase (GUS) and proteases (e.g., trypsin). Corn seed is regarded as an ideal platform for the production of industrial proteins/enzymes because corn has the largest annual grain yield and relatively high seed protein content (10%), thus offering the highest potential recombinant protein yields per hectare. Corn-produced enzymes such as cellulases and hemicellulases involved in biomass conversion to produce biofuels such as ethanol are currently interesting candidates for commercialization [5].

1.3 Biopolymers

Recombinant biopolymers are spider silk proteins, elastin-like polypeptides (ELPs), collagens and plant gums. The spider silk proteins (spidroins) that are modular fibrous proteins containing highly repetitive amino-acid sequences consisting largely of glycine and alanine [9].Spider silk fibers spun from these spidroins are regarded as one of nature's most extraordinary materials with exceptional flexibility, elasticity, and toughness—three times as strong as Kevlar and five times as strong as steel [10].Plants offer a more efficient and cheaper production platform than bacteria for production of recombinant spidroins. ELPs that are comprised of the repetitive pentapeptide sequence (VGVPG), which mainly serves as thermally responsive tags for the non-chromatographic purification of recombinant proteins [11]. ELP tags werefound to significantly enhance the production yield of a range of different recombinant proteins in plant leaves [12].

2. Plant expression systems

The rapid development of plant genetic engineering technologies has expanded the diversity of well-established plant based bioproduction systems for recombinant proteins [12]. The used plants have advantages and disadvantages (Table 1). Each of the systems has its own strengths and weaknesses, which are described here.

Table 1 Comparison of advantages and disadvantages associated with the three major types of plant.

	Advantages	**Disadvantages**
Seed crops	✓ Grown in open-fields, ✓ the economy of scale, ✓ established agricultures practices, ✓ Seed proteins do not degrade at ambient temperature ✓ Stable for long term storage ✓ seed has superior aspects as a mucosal delivery vehicle for oral peptide／protein therapeutics ✓ improved consistency of protein product with the use of controlled bioreactors which are less prone to biotic- and abiotic-induced variations that commonly hamper field/greenhouse grown plant-based protein production	✓ lower biomass yields ✓ They have long growth cycle ✓ Not suitable for transient expressions ✓ In the pollination period, the potential transgene could escape into the enviroment ✓ Issues of gene silencing of nuclear transgenes limit production performance
Leafy crops	✓ High biomass yield, ✓ possibility for multiple growth cycles per year, ✓ established agricultural infrastructure like seed systems ✓ less risk of pollen spreading (flowering can be prevented) ✓ low-up front capitilazation costs	✓ using transient expression ✓ applied specifically to expression of monoclonal antibodies (mAbs), vaccines, and other therapeutics products ✓ high water content, ✓ Non storage stability of harvested biomass, ✓ recombinant protein stability which does not allow decoupling of upstream and downstream processing. ✓ phenolic compounds, and chlorophyll-derived pigments that could pose difficulties during downstream processing
Aquatic plants	✓ safe ✓ fast-growing ✓ easy to grow and harvest ✓ has a high protein content ✓ offers an attractive system for oral vaccines	✓ contain small amounts of phenolics (primarily ferulic and coumaric acids), phytic acid, lipids, and lectins that may interfere with protein purification ✓ Difficulty in culture scale up ✓ Higher capital investment

The selection of the promoter is the first thing to consider before making the construct. Promoter elements can dramatically affect the level of messenger RNA and, thus, influence the accumulation of the protein[14]. To obtain high transcription levels, many constitutive promoters have been used to drive the transgene. The most widely used promoter is the cauliflower mosaic virus (CaMV) 35S promoter [15], but it is much weaker in monocotyledonous plants. In dicotyledonous plants, strong constitutive promoters, such as rice actin-1 or maize ubiquitin-1, are more frequently used for expressing foreign proteins [16].

Codon usage should be considered when a transgene is expressed in a host plant with different codon use frequency to achieve optimal expression levels. Nowadays, codon optimization is a standard practice for transgene expression. Many codon optimization programs are available on the Internet based on the analysis of sequences available for a given species[17,18].

Subcellular targeting is an alternative strategy to enhance the expression of exogenous proteins by avoiding protein degradation caused by proteases in the host plant. Recombinant proteins can be targeted to the cell wall, the vacuole, the mitochondria, and the chloroplast by fusing with different targeting signal. If a targeting signal sequence is not designed, the recombinant protein may be released in the cytoplasm where the protein could be degraded by proteases [19]. The endoplasmic reticulum (ER) is an ideal target compartment for recombinant proteins. The ER retention sequences, such as KDEL (Lys-Asp-Glu-Leu) or HDEL (His-Asp-Glu-Leu), are used to keep recombinant proteins within the endoplasmic reticulum. This strategy has significantly enhanced the expression of foreign proteins in transgenic plants[20].

Protein fusion approaches are effective for boosting the expression, increasing solubility and stability, and facilitating isolation and purification of recombinant proteins [21]. Zera is maize seed storage protein γ- zein derived proline-rich N-terminus domain, which has self-assembling and protein body formation properties [14]. Another successful fusion partner example is elastin-like polypeptides, which are artificially-designed biopolymers.

In the maize *o2* mutant, non-zein proteins have increased, while zeins are reduced significantly, resulting in improved maize seed nutritional quality [22]. In cotton, the RNAi mediated knock-down of two key fatty acid desaturase genes leads to the increase of high-oleic and high-stearic cotton seed oil, essential fatty acids for human heart health [23]. In soybean, while the expression of α and α' subunits of β-conglycinin is suppressed by sequence-mediated gene silencing in seeds, another soybean seed storage protein glycinin is increased to balance the total seed protein [24]. A strategy is raised to improve the nutritional quality or to enhance the expression of recombinant proteins in seeds, using the gene silencing mechanism to reduce a major seed protein gene [25].In *Arabidopsis thaliana*, a transgene *arcelin5-I* from *Phaseolus vulgaris* increased up to 15% of the total soluble protein of the seed when the endogenous seed storage protein 2S albumin is silenced by antisense. Two major maize seed storage proteins, α- and γ-zeins, have been silenced by antisense or RNAi methods [26]. Non-zein proteins increased in these transgenic maize lines and seed nutritional quality improved high-lysine maize [27].

2.1 Stable Seed based systems

Seeds have used many applications in the area of molecular farming. Seed provides its genotype with powerful selective advantages because of its flexibility in the face of a changing environment. They provide a plant with the ability to germinate and grow under nutrient-limited conditions because of the availability of storage products. Seeds allow germination to take place under environmental conditions, which most favour the seedling's survival[28]. Seeds are naturally designed for protein storage for long periods of time without or with very little degradation [29]. Seed dormancy is a very important property of many seeds. The ability of a seed to go into a quiescent state for long periods or until an appropriate environmental signal is detected provides a substantial survival advantage for many wild species. The stability of the recombinant protein and the dormancy of the seed also allows for a complete decoupling of the cycle of cultivation from the processing and purification of the protein. Current Good Manufacturing Practices (cGMP) manufacturing, this is extremely helpful as it permits the establishment of such concepts as a 'master seed bank' and also allows for the establishment of quality-based release criteria for the seeds as a precursor. Seeds can be stored after harvest without cooling or immediate isolation that are required for leafy tissues [28]

Proteins expressed in seeds are generally protected from proteolytic degradation, and storage upwards of three years at room temperature (longer with cold storage) resulted in minimal loss of protein activity. Seeds have a relatively lower biomass and higher cross-contamination risk by pollen drift for non-self-pollinated plants. However, considering the stability of foreign proteins, post-harvest processing, and overall cost, the seed-based platform is still more suitable for many recombinant proteins production on a large scale [30]. Localization of storage products at the cellular level is a major determinant of accumulation levels of the product as well as the potential stability of the product during prolonged periods of dormancy. For example, a recombinant protein targeted to the cytoplasm may accumulate at moderate levels, but never satisfactory levels, whereas the same protein in a configuration which targets the secretory pathway can accumulate at high levels suitable for economic production [28]. Recombinant protein accumulation in seed has been shown to mimic the abilities of naturally occurring proteins. So, for example ,in rice, the use of storage protein promoters to drive expression of genes for mammalian proteins such as human lysozyme has resulted in an average expression level of 13%–14% of total soluble protein[31]. Neither seed nor leaf extraction can be performed at a large scale under aseptic conditions, most seeds can be subjected to a surface 'sterilization' technique, which reduces bioload to industrially accepted standards for a raw material. This is difficult if not impossible to do with a large mass of leaves without damaging the mesophyll cells, which carry the bulk of the recombinant product. One of the disadvantage in seed-based expression systems is speed to proof-of-concept, or in the case of recombinant proteins required in relatively smaller volumes, the time-to-product may be preclusive. [28].

The first plant-derived commercialized product was produced in maize derived avidin and trypsin. Hovewer, oil-seeds are emerging as a promising platform for recombinant protein production due to their inherently low associated

proteolytic activity and simplified protein isolation via oil body separations. During the past decade, plants have emerged as promising biopharming systems for commercial production of pharmaceutical proteins (Table 2). SemBioSys Genetics (Calgary, Canada), which has developed the oleosin-fusion platform, in which the target recombinant protein is produced as a fusion with oleosin and accumulates in safflower oilbodies (www. sembiosys.com/). Ventria Biosciences and Meristem Therapeutics, which have invested in field-grown rice for the production of the human proteins lactoferrin and lysozyme (www.ventria.com/). ORF Genetics Ltd., based in Iceland, has targeted barley grain as the expression site for a number of human cytokines and growth factors (www.orfgenetics.com/).

Table 2 Plant-derived pharmaceutical products in clinical development manufactured.

Product	**Platform-plant**	**Comments**
Escherichia coli heat-labile enterotoxin (LT-B)	Transgenic potato tubers	[32]
Norwalk virus coat protein		[33]
Norwalk virus capsid protein		[34]
Rabbit haemorrhagic disease virus (RHDV) VP60		[35]
HBsAg		[36,37]
Combination cholera⁄ E. coli⁄ rotavirusvaccine		[38]
Human papilloma virus E7 and L1 proteins		[39,40,41]
Newcastle disease virus envelope proteins		[42]
ZMapp	Tobacco leaves	[43]
Immuno adhesin (DPP4-Fc)	Transgenic tobacco	[44]
Hepatitis B surface antigen (HBsAg)		[45]
Human growth hormone	Tobacco and sunflower	[46]
Taliglucerase alfa; Recombinantglucocerebrosidase (prGCD)	Carrot cellculture	[47]
Therapeuticenzym (Glucocerebrosidase (UPLYSO)		[48,49]
Human Vascular endothelial growth factor (VEGF)	Barley	[50,51]
Trypsin	Transgenic maize	[52]
Soybean derived monoclonal antibodies(mAbs)	Soybean	[53]
Human acidic fibroblast growth factor 1 (FGF-1)	Salviamiltiorrhiza	[54]
Therapeutic protein (Apo-A1 ^{Milano})	Saffflower	[55]
Antibody(Anti-CD20 mAb (BLX-301)	Lemna (Duckweed)	[56]
Human serum albumin	Flax	[57]
Lactoferrin	Transgenic rice	[58,59]
Collagen	Transgenic maize	[60]
Therapeutic enzyme		[61]
Human intrinsic factor	Transgenic Arobidopsis	[62,63]
Pandemic and seasonal influenza vaccines	N. benthamiana	[64]
Hepatitis B surface antigen	Transgenic potato Transgenic lettuce	[65,66]
Rabies glycoprotein	Viral vectors in spinach	[67]
Various anti-idiotype IgG antibodies clinical trial NCT01022255	N. benthamiana	[68,69]
hGAD67/ 65 mut	Arabidopsis Thalina Petunia	[70]
2 G12	Maize, Arabidopsis Thalina	[71,72,73]
NVCP	Lettuce	[74]
	Potato	[75]
	Tomato	
hGAD65	Carrot	[76]

2.2 Stable Leaf based systems

Tobacco is the leading leaf-based protein expression system for commercial products [1]. The first commercial products were tobacco-derived secretory IgA. Tobacco is genetically well studied and easily manipulated, is classified as a non-food/non-feed crop, produces high biomass (upwards of 300 tons per acre) and is one of the best studied platforms to date for expressing recombinant biopharmaceuticals. [77]. Many other leafy crops have been used for stable expression of proteins including lettuce, alfalfa and clover. The use of crops like alfalfa has additional benefits; it is a perennial that fixes nitrogen and displays notable homogeneity of N-glycosylated recombinant proteins[78].The process of extracting

recombinant proteins from leafy plants does not require additional operations, such as seed grinding, soaking, and de-oiling [30], but leafy plants have high water content and low storage stability [3]. Leaf-based systems are able to produce a high yield of recombinant proteins, this yield may be offset by an increased instability of expressed proteins in metabolically active tissues, such as leaves [3].

Both nuclear and plastid integration have been used when expressing recombinant proteins in leaf tissue. The choice of gene integration within the plant cell is generally dictated by the post-translational requirements of the target protein. For glycosylated proteins, nuclear integration of the transgene is needed to enable proper processing of the protein in the endomembrane system [4].There are many cases of recombinant proteins in leaves having associated stability and accumulation issues. Product yields in field-grown materials can be highly variable due to environmental impacts (both biotic and abiotic stresses) leading to increased consideration of growth in more controlled conditions (e.g., under plastic or in greenhouses), especially for pharmaceutical applications [13]. For example, algae possess a number of advantages over transgenic plant systems for the production of recombinant proteins. They can be grown in contained bioreactors, reducing the risk of contamination of the production system by airborne contaminants, and also protecting the environment from any potential flow of transgenes into the surrounding ecosystem. Growth in containment also greatly reduces the potential for loss of the crop due to predation or pathogen attack[79]. Robert [80], assessed the potential of methyl jasmonate (MeJA) as a generic trigger of recombinant protein enrichment in Nicotiana benthamiana leaves before harvesting. They found that overall the ability of MeJA trigger ribulose 1,5-bisphosphate carboxylase oxygenase (RuBisCO) depletion and recombinant protein enrichment in N. benthamiana leaves.

2.3 Transient Expression System

Transient expression systems, mediated by recombinant viral or plant binary vectors, are being increasingly used for the expression of biopharmaceuticals in plants due to speed, high protein yields, and regulatory considerations. During this process, foreign genes are typically introduced into leaf tissue of intact plants (generally non-transgenic) by vacuum infiltration of engineered Agrobacterium (containing gene(s) of interest within T-DNA with or without additional virus-derived components). Recombinant protein production (based on extrachromosomal gene expression within the plant cell) is initiated in the leaves within 24 h and continues for several days (Agro-mediated) to several weeks (viral mediated) depending on vector and protein. While chromosomal integration is possible, it occurs at considerably lower frequency in comparison to the number of cells transiently expressing the desired gene [81].

There are two basic approaches for plant transient expression based on the mechanism by which the transgene is transferred into the plant cell: viral-mediated or plant binary vector-based [82]. The accumulation of recombinant proteins in plants occurs within a shorter time, typically 2–4 days post-infiltration, with recoveries typically ranging from ~0.1 to 180 μg/g fresh leaf depending on the gene of interest by conventional" plant expression vectors, [83]. Plant viral vectors also use the same Agro-infiltration system to introduce DNAcopies into the plant cell. This transient expression process generally produces higher levels of recombinant protein with yields reported to be as high as 5 mg/g of fresh leaf for GFP (green fluorescence protein gene) [84. However, recombinant protein expression by this process takes up to 14 days, which can present issues for proteins prone to protease degradation and instability. Recombinant protein production using transient expression is now being mobilized to large scale by industry such as pharmaceuticals and vaccines. The production of large quantities of recombinant protein offered by transient plant expression systems, coupled with use of current technology to increase yields and many technical promising solutions seem to favorably compare with mammalian or insect cellbased systems in quality, cost, and scale. [13].

The production of vaccines using a transient plant expression system has been developed rapidly, somewhat due to the recent outbreak of avian and swine flu, which led to the development of seasonal and pandemic influenza vaccines.

2.4 In Vitro Culture System

In vitro culture systems are characterized by the fact that plant biomass is cultured in confined bioreactors under sterile conditions for large-scale production of recombinant proteins.

Like microorganisms, undifferentiated clusters of plant cells (callus) can be dispersed and propagated in a liquid medium to generate stable cell suspension cultures, which can retain the same production capacity as whole plants. Increased concerns about regulatory compliance and product safety of mammalian systems recently have renewed interest in plant cell cultures as an alternative production platform for complex pharmaceutical proteins. These systems provide cGMP-compatible production environments more acceptable to the established pharmaceutical industry with added benefits of complex protein processing compared to bacteria and yeasts, and increase safety compared tomammalian cell systemswhich can harbor human pathogens. [13].

Hairy roots are generated by infection of plants with Agrobacterium rhizogenes that harbors a large root-inducing plasmid [85]. Hairy roots can be axenically cultured in a controlled environment suitable for the production of high-value pharmaceutical proteins under cGMP requirements. In addition, the possible extracellular secretion of expressed proteins from cultured hairy roots, or rhizosecretion The advantages of rapidly growing hairy roots over suspension cells include long-term genotype and phenotype stability, efficient productivity and the ability of hairy roots to grow on

hormone-free medium. [13].The challenges for large scale culture of hairy roots because they have a tangled, filamentousmorphology and often possess unique physiological characteristics.

3. Strategies of recombinant proteins

There are several strategies for the primary recovery and purification of recombinant proteins from seed, leafy, and bioreactor-based platforms. They are upstream processing and downstream processing. These strategies will be briefly reviewed below:

3.1 Downstream processing

Downstream processing efficiency is influenced by the recombinant protein concentration, the complexity of the plant extracts or cell-free culture media, and required final product purity. Downstream processing can be divided into two phases: primary recovery and purification [86].

The primary recovery steps for leafy- and seed-based production systems consist of product release from the biomass by homogenization or aqueous extraction; solid–liquid separation; and conditioning, pretreatment, and clarification. Some primary recovery unit operations, such as centrifugation, cross-flow filtration, and dead-end filtration, are multi-functional, and their strategic applications are defined by the extract composition, particle size, extract properties, and product stability [1].

Fractionation uses established processing methods, such as dry milling, dry fractionation, and wet milling, to reduce total processing volume and solids content, to enrich the recombinant protein, and to generate co-product revenues for seed-based systems [87].

The protein release/extraction process is a critical recovery step because it dictates the total extract volume, recombinant protein concentration and purity, and the type and quantity of impurities that have to be removed during purification [88]. Ground seed is extracted with low-shear mixers using 4 to 5 L of buffer per kg dry seed. Extraction of oil bodies for oleosin-fusion technology requires much greater water-to-tissue ratios [89]. The ratio of recombinant protein to native protein is another critical factor for reducing the purification burden that can be easily manipulated by adjusting the extraction buffer pH and ionic strength and plant-tissue-to-buffer ratio[90]. For example, extraction of corn seed with pH 3 buffer instead of pH 9 bufferresulted in an eight-fold decrease in native protein concentration and a two-fold decrease for the same pH range for tobacco leaves [91]. Low pH extraction (pH 3) allowed easier purification of collagen and aprotinin from transgenic corn extracts [92].

Centrifugation is a common method for solids removal and/or clarification of plant extracts and homogenates because continuous or semi-continuous processing is feasible, the process is easy to scale up, and can handle a variety of solid–liquid suspensions.

Plant extracts and plant cell homogenates have a unique composition of native proteins, cell metabolites, and process-derived impurities, and the presence of these potentially harmful impurities may require distinct handling or conditioning of the extract. The purpose of conditioning is to maximize product binding by capture chromatography, reduce binding of plant components to the recombinant protein, and stabilize extracts for purification. Traditional conditioning methods, also referred to as pretreatment methods, for any protein production platforms include adjustment of extract or media pH, ionic strength, buffer composition, and volume reduction by cross-flow filtration. [1]. Several approaches have been implemented specifically for pretreatment of plant extracts including aqueous two-phase partitioning, adsorption, precipitation, and membrane filtration.

The purification phase usually starts with a capture step to concentrate the recombinant protein and most importantly, remove critical plant impurities that would be detrimental to protein yield, quality, and/or purification efficiency [93]. Purification of plant-derived proteins, similarly to other protein production systems, is dependent on protein properties and host cell impurities, and utilizes similar strategies for purification development as other heterologous expression systems. There are very few studies reporting purification processes for plant-derived recombinant proteins that have reached clinical trials or been scaled-up to commercial production. The reason for this is twofold. First, relatively few companies have reached later stages of clinical trials (Phases II and III) to have established robust, at-scale downstream processes and, second, newly developed processes are often deemed proprietary [28].

Capture chromatography resins have two primary functions, concentration and partial purification, and should be inexpensive, resistant to chemicals needed for resin regeneration, and able to retain capacity and selectivity over multiple cycles. Resin selection is determined by recombinant protein properties such as charge, hydrophobicity, and biospecificity. Selecting a resin based on the property most unique to the recombinant protein compared to the host system can improve purification efficiency by increasing binding capacity and/or product purity. Once suitable chromatography resin functionality is determined (cation/anion-exchange, affinity, hydrophobic), various resins with different particle sizes, surface areas, and resin backbones need to be screened along with binding conditions, such as pH and ionic strength. [1].

The manufacturing cost for plant-produced proteins consists of upstream (biomass production) and downstream purifications costs. The downstream processing costs are also affected by the ease of product recovery, the complexity

of clarified plant or cell culture extracts, protein stability, and required purity [94]. The support activities, such as process and cleaning validation, buffer preparation, equipment cleaning, and quality control and quality assurance, could be a substantial fraction of operating costs. From a downstream cost perspective, several evaluation criteria could be applied for selecting the best system that matches product characteristics and allows overall manufacturing cost reduction. In addition to protein expression level and stability, the following criteria should be considered: biomass yield and storage stability (allowing processing flexibility and decoupling), selection of off-the-shelf purification tools, biomass disposal cost, and byproduct revenues (e.g. biomass and starch conversion to energy) [1].

3.2 Upstream processing

The commercial and economic success of any plant-based production platform for recombinant proteins depends on the product yield, since the costs of upstream production relate to the amount of biomass produced, and the greater the amount of product per unit of biomass the better.

The subcellular localization of a protein contributes to its stability, so further yield improvements have been achieved by adding targeting sequences, such as a signal peptide to allow secretion (into the medium for plant cells, or to the apoplast in whole plants), a signal peptide and a KDEL/HDEL tetrapeptide so that secreted proteins are retrieved to the ER, or a signal peptide and a transmembrane domain so the recombinant protein is concentrated in the membrane fraction[95]. Another strategy involves the expression of recombinant proteins as fusions with stabilizing sequences, such as the elastin-like peptide repeat, which not only increases yields but also provides a convenient extraction method known as reverse transition cycling (reversible temperature-dependent precipitation) [12].

The initial step in the process usually involves milling of the tissue in either a dry state or together with an aqueous extraction buffer. In some cases, this may be preceded by a dehulling step or separation of endosperm and germ (corn) to remove a portion of the biomass that is largely devoid of the recombinant protein. If a dry milling process is employed, it is usually followed by a separate aqueous extraction step. The method of choice may be dependent on seed type, target protein and nature of the process. For an undisclosed protein expressed in corn, it was found that wet milling resulted in product losses and so the two-step dry milling and extraction process was preferable [96]. Extraction of the tissue is generally followed by some form of filtration or centrifugation to remove solids that can represent 5%–30% of the total mass of the slurry. At this stage, in most seedbased processes, it is often desirable to perform a concentration step to reduce volumes prior to advancing into chromatography. The safflower oilbody process differs here in that the extract consists of an emulsion in which the recombinant protein is bound to seed oilbodies. The oilbodies and bound protein are separated from the majority of endogenous seed protein through a series of flotation–centrifugation steps. The washed oilbody fraction is then treated to either elute or cleave off the recombinant protein and the oilbodies removed [28].

4. Legal regulations

The potential to use agronomically important food/feed commodity crops and other seed crops for non-food/feed GM-based applications such as biofuel enzymes or pharmaceuticals raises several regulatory issues for both regulatory agencies and consumers. Regulatory compliance for field growth (e.g., USDA APHIS/EPA) will encompass concepts of product segregation and stewardship. Crops expressing therapeutics or vaccines will likely remain under regulatory oversight for the life of the product and require containment protocols and documentation at each step in the process. In contrast, for industrial enzymes which are/will be produced at large scale, producers will likely seek deregulation. One of the regulatory considerations for food-based grains is that while there is the need to segregate the bioproduct source grain from the food/feed supply chain, they do have a G.R.A.S. status from the US Food and Drug Administration (FDA) which may reduce downstream hurdles linked with pharmaceutical use of these plant-based products [97].

Both US (FDA) and European (EMEA) regulatory authorities have recently published guidance documents on the production of therapeutic proteins from genetically engineered plants providing a framework for manufacturing in these systems [98,99]. The documents cover all aspects of the process from generation of the bioengineered plant through propagation and final purification. As the part of the process that differs most from that of conventional cell-based systems, considerable attention is given to production of the plant material used for manufacturing.

In 2005, the World Health Organization (WHO) conducted an ‘informal consultation on scientific basis for regulatory evaluation of candidate human vaccines from plants’[100]. In its report, the WHO recommended that the guidelines on Good Agricultural and Collection Practices (GACP), which are typically applied to herbal plants, should also be applied to plants producing biopharmaceuticals. A report of quality-control methods for medicinal plant materials recommended tests to assess the identity, purity, and content of biopharmaceutical plant materials. [101].

Since 2008, the United States Department of Agriculture (USDA) has approved field release of transgenic seeds expressing human lysozyme, lactoferrin, and serum albumin in rice, apolipoprotein in safflower, and hepatitis B surface antigen and brazzein in corn.

5. Conclusions

Since plant cell cultures are inherently simpler, cheaper and safer than mammalian cell approaches and support eukaryotic processing (e.g. complex glycosylation) absent in microbial systems, this technology has continued to attract attention as an alternative host system. For the process of recombinant proteins, most suitable subcellular compartment or organelle should be selected for easy recombinant protein extraction; protein product and plant extract properties should be match for optimal purification; for oral vaccines, plants should be selected with GRAS status, it should benefit chloroplast expression for high expression levels.

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