

Plants as Expression Systems for Recombinant Proteins

ABSTRACT

Plants provide a wide range of biological molecules that are needed in research, medicine and industry. Currently, there is a growing interest in the use of plants as expression systems for the production of recombinant proteins using different host cells. The synthesis of proteins in plants is safe, economical and can be scaled up compared to the microbial and mammalian expression systems. Despite the fact that plant systems are widely recognized for producing recombinant proteins, there are certain improvements that should be addressed before the system is considered commercially viable. Such challenges include the need to identify promoters that would provide comparable yield to other systems, enhance protein stability and recovery. In this review, we describe the production of some plant based proteins, challenges and advantages of plants systems.

Key words: Microbial, mammalian, expression systems, promoters

1 INTRODUCTION

Plants are increasingly being considered as alternative expression systems for the production of proteins. The interest in plants is due to their ability to be easily transformed as cheap sources of protein. Currently there are several biotechnology companies that are actively developing, field testing, and patenting plant expression systems [1]. Traditionally plants have been used as sources of many biologically active molecules including proteins. These molecules are required for research, medicine and industrial applications however their extraction from natural sources is usually difficult and expensive [2]. Target recombinant proteins are usually expressed in transgenic plants with desired characteristics. Transgenic plants are genetically engineered through breeding techniques using recombinant DNA technology. Generally transgenic plants are produced in two ways; first by using *Agrobacterium* mediated transformation, particle bombardment and other standard transformation techniques. There are several *Agrobacterium* transformed plants producing recombinant proteins including *Nicotiana tabacum*, a model expression system for proteins [3]. Secondly, non transgenic plants are infected with recombinant viruses that express transgenes during their replication in the host [1].

The two commonly used host viruses are tobacco with tobacco mosaic virus and cowpeas with cowpea mosaic virus [1, 4]. Despite the product yield being typically similar to those of the transformed plants, they are always higher [5]. Expression of proteins in transgenic plants involves cloning and expression of the gene of interest in plant cells, regeneration and selection of the plants, recovery and purification of the protein and finally characterization of the final product [6].

There are general procedures in obtaining and purifying desired proteins from plants [7]. Irrespective of the plant species, usually the plant source material is first ground (seeds) or

homogenized (green tissue) followed by cold buffer extraction and centrifugation. However there are challenges encountered during extraction of proteins like those posed by the plant enzymes. Kusnadi et al. [6] described the problems as inefficiency of protein extraction, possible structural modification of the product due to secondary reaction with phenolics and proteolytic degradation. The quantity of phenolics varies among different plants but green plant parts contain more phenolics than seeds. The principle of plant protein purification is similar despite the source of the target protein and the choice of purification sequence does not depend on the scale and tissue from which the plant has been extracted [7]. Protein scale up process may further lead to problems such as reduced extraction, purification yields and activity loss due to foaming shear [8]. Recombinant proteins are usually isolated together with plants natural proteins to assess the accumulation levels, establish protein functionality and to confirm the amino acid sequence. Based on the type of the recombinant protein and transgenic plant, different extraction methods are always used. This review describes some plant based proteins, challenges and advantages of plants as expression systems for proteins.

2 Some recombinant proteins expressed in plants

2.1 Phytase

Recombinant proteins such as phytase from *Aspergillus niger* have successfully been expressed in transgenic plants at higher levels. Phytases are enzymes that release phosphates from phytates, a major form of phosphorus (P) in animal feeds. Production of phytase from microbial sources is associated with high production and processing costs [9]. Therefore transgenic plants offer alternative expression system for phytase for commercial use. Phytase genes from microbial sources have been expressed in many plants including tobacco, canola, soybean, wheat, rice, alfalfa, sesame, *Arabidopsis*, maize, *Trifolium repens*, *Medicago truncatula* and potato [10-12]. The technique involves fusing the fungal phytase gene (*phyA*) to a plant endoplasmic reticulum and placing it under the control of the constitutive 35S cauliflower mosaic virus (CaMV) promoter in a binary transformation vector [9]. The gene is inserted into the tobacco genome by *Agrobacterium*-mediated transformation [13]. Several studies have demonstrated that transgenic plants contain sufficient phytase activity that could replace microbial phytases [14, 15]. The plants could reduce downstream processing and formulation costs involved in the commercial production of phytases and serve as bioreactors.

2.2 Hirudin

Hirudin is an anticoagulant protein that was initially isolated from the salivary gland of leech, *Hirudo medicinalis* and is used for treating thrombosis [16]. Despite hirudin being produced mostly by recombinant bacteria and yeast, plants such as oilseed rape, tobacco, and Ethiopian mustard have been engineered to produce the same protein [17]. The fusion of hirudin genes with oleosin genes has reduced the cost of protein purification making commercial production easier and cheaper [18]. The plant oleosin-hirudin fusion protein is expressed and accumulated in the seeds and separated from other seed proteins by flotation and centrifugation [1]. The method ensures that hirudin becomes active only after harvesting thus

limiting environmental exposure [1]. It is therefore evident that the fusion of the oleosin and hirudin has a potential for commercial production of the protein in transgenic plants.

2.3 Enkephalin

Enkephalin is one of the earliest transgenic peptides in plants with potent painkilling effects. The peptide is released by neurons in the central nervous system and cells in the adrenal medulla. The method of expression involves insertion of DNA coding sequence into the gene coding for a seed storage protein called 2S albumin [19, 20]. The 2S albumins are amongst the smallest seed storage proteins with conserved and variable sequences derived from group of structurally related genes [21]. The strategy employed to produce Enkephalin involves substituting part of the variable sequence with DNA sequence coding for the five amino acid neurohormone. The DNA construct is then flanked on both sides by codons coding for the tryptic cleavage site. The expression of the altered 2S albumin gene results in the production of a hybrid storage protein containing Enkephalin sequence [22]. The sequence subsequently released from the altered protein tryptic cleavage and purified by High Performance Liquid Chromatography (HPLC).

2.4 Thaumatin

Thaumatococcus is a sweet-tasting protein that is widely used in the food industry. The peptide is isolated from the fruit of the African katemfe (*Thaumatococcus daniellii* Bennett) [23]. Besides the sweet taste, thaumatin enhances certain flavours while masking others, binding specifically with taste receptors [24]. The antifungal activity and sweet taste of the protein makes it a good candidate for use in genetic engineering for producing disease tolerant crops with modified tastes. The protein has naturally been extracted from plant fruits for commercial production of sweeteners and flavor enhancers. Attempts for commercial production of thaumatin in microorganisms has resulted in low product yields reducing the economic feasibility for large-scale production [23]. Thaumatococcus gene has successfully been expressed in potato hairy roots and cucumber using an *Agrobacterium rhizogenes*-based approach [25]. Thaumatococcus expression in the plants was confirmed at protein level and sensory evaluations [26].

2.5 Monellin

Monellin is a sweet protein consisting of two noncovalently bound polypeptide chains, A and B [27, 28]. The protein is extracted and purified from the West African plant *Dioscoreophyllum cumminsii* and sweeter than sugar with a slow onset of sweetness and a lingering after taste [27, 29]. The A and B chains have a secondary structure containing 44 and 50 amino acids, respectively [30, 31]. Native Monellin protein is pH and temperature dependent with an optimal taste ranging between pH (2 and 9) [32]. Higher temperatures above 50 °C denatures the protein and causes the separation of the units leading to the loss of sweetness [33]. The instability challenges of the protein have been addressed through downstream processes [27]. Further, thermal stability and quality of sweetness of the sweet tasting protein has been increased through site-directed gene mutagenesis and protein modification [34]. The technique involved obtaining mutants by alanine substitution of amino acids at different sites in the Monellin protein [35]. Results showed that the thermal stability

and sweetness of the natural Monellin protein can be improved by gene mutation technique [36]. These findings demonstrate the potential of commercial production of Monellin as an alternative source of sugar.

2.6 Papain

Papain commonly called the vegetable pepsin is a cysteine protease isolated from the latex of the green fruit and leaves of *Carica papaya* [20]. The protein consists of a single polypeptide chain with three disulfide bridges and a sulfhydryl group that is necessary for its activity [37]. The enzyme is commercially used as a meat tenderizer due to its ability to dissolve tissues without damaging the cells [38]. During the tenderization process, the proteolytic activity of papain is directed at collagen, the major structural protein in animals, about a third of all vertebrate protein. The collagen present in the connective tissue and blood vessels is responsible for the toughness of the meat and therefore hydrolysis of the peptide bonds in collagen is achieved either by boiling or papain activity [20]. Proteolytic activity or boiling degrades the tough insoluble collagenous fibrils yielding soluble polypeptides of lower molecular weight. The enzyme is injected directly into the blood stream of animals before slaughter to achieve even distribution. Due to the thermal stability of papain, it is able to retain its proteolytic activity during the initial stages of cooking. Industrially, papain is used in clarifying beer, clarifying preservation of spices, contact lens cleaners, detergents, pet food palatability, digestive aids, blood stain remover, blood coagulant and cosmetics [20, 39]. Other uses include aiding protein digestion in chronic dyspepsia, gastric fermentation, gastritis, removal of necrotic tissue, preparation of tyrosine derivatives for the treatment of Parkinsonism, preparation of tetanus vaccines, skin cleansing agents, acne treatment [40]. The demand for more stable, highly active and specific enzymes such as papain is growing rapidly [41, 42].

Currently, different plant species are being exploited to determine their potential in expressing therapeutic proteins of interest. Examples include the production of human erythropoietin and transferring in *Nicotiana benthamiana*, α -L-iduronidase in *Arabidopsis thaliana* and *Brassica napus* and anti-HSV glycoprotein B in soybean [43, 44]. The expression of a recombinant human glucocerebrosidase (GCD) in transgenic carrot cells grown in suspension culture has been described to be capable of producing glycoproteins [45]. GCD in carrot cells is structurally and highly homologous to the commercial Cerezyme displaying comparable enzymatic activity and uptake into macrophages [46].

There are several other commercial proteins that are produced in plants primarily to enhance their agronomic performance. As indicated in Table 1, these plants are able to express and accumulate proteins of potential industrial applications that were originally produced by other systems [6]. However there are many factors to be considered when selecting transgenic plant system for production of proteins. The critical factors include the level of protein accumulation, germplasm, handling, processing of transgenic plant material, and protein purification can influence quality, quantity, purification and the cost of the final product.

Recombinant Protein	Initial Source	Host
a-Amylase	<i>Bacillus licheniformis</i>	Tobacco
Aprotinin	Bovine	Corn
Avidin	Chicken	Corn
Chymosin	Calf	Tobacco, Potato
Cyclodextrin glucanotransferase	<i>Klebsiella pneumoniae</i>	Potato
Erythropoietin	Human	Tobacco
Glucoamylase	<i>Aspergillus niger</i>	Potato
β Glucuronidase	<i>Escherichia coli</i>	Corn
Growth hormone	Trout	Tobacco
Heat-labile enterotoxin B	<i>Escherichia coli</i>	Tobacco, potato
Hepatitis B surface antigen	Hepatitis B virus	Tobacco
γ - and k-chains hybridoma	Mouse	Tobacco
b-Interferon	Human	Tobacco
Levansucrase	<i>B. subtilis</i> , <i>Streptococcus mutans</i>	Tobacco, potato
Lysozyme	Chicken	Tobacco
Malarial epitopes	<i>Plasmodium</i>	Tobacco
Phytase	<i>Aspergillus niger</i>	Tobacco
Xylanase	<i>Clostridium thermocellum</i>	Tobacco
Serum albumin	Human	Human
Ricin	Castor bean	Castor bean

Table 1: Industrial recombinant proteins expressed in transgenic plants

3 Advantages of Plant Systems

Several studies have demonstrated that recombinant proteins from plants are superior in terms of storage and distribution compared to animals and animal cell cultures [47, 48]. The expression of proteins in plants is considered better than other systems like microbes due to their safety, cost, and protein complexity and require less time [49]. Due to practical, economic and safety benefits compared with conventional systems, the use of plants for large scale synthesis of proteins is gaining wider acceptance in the world [47, 50, 51].

There are minimum risks of contamination with pathogenic microbes since no known incidences plant diseases have been found to be pathogenic to humans [47]. Cultivation of plants is less complex compared to microbes or mammalian cell cultivation that is extremely expensive and requires good laboratories with competent technical and scientific expertise. Plant proteins can be expressed in the edible parts such as roots or leaves that are consumed raw thus eliminating the need for purification [52]. Plants are more economical in protein production compared to mammalian cell culture and microbial fermentation depending on the protein of interest, product yield and crop used [53].

187 Proteins of interest can be expressed in various targeted cells or tissues like seeds or tubers
188 where they are more stable, easy to transport with no need for refrigeration [52]. Industrial
189 production of plant proteins can be achieved through increased cultivation of more crops.
190 Plant systems are capable of performing most of the post-translational modifications required
191 for protein stability, bioactivity and pharmacokinetics [54]. Studies have demonstrated that
192 glycosylation, targeting, compartmentalization of simple proteins such as interferons and
193 serum albumins have successfully occurred in plants [47]. In contrast to microbes, plants are
194 capable of expressing and assembling antibodies in their active form [55].

195

196 **4 Limitations of Plant Systems**

197 Despite the increasing preference of plants as expression systems, their use for the production
198 of therapeutic proteins still remains at developmental research [52]. Such limitations arise
199 from low level of expression, accumulation and incorrect post-translational modifications of
200 the recombinant protein [56]. Plant proteins lack the terminal galactose and sialic acid
201 residues commonly found in animals, and have α -(1,3) fucose and β -(1,2) xylose which are
202 absent in mammalian systems. Glycoproteins from plants can therefore lead to
203 immunogenicity and affect pharmacokinetic properties [57]. Economically, higher plants are
204 not preferred systems for expressing commercial proteins for many reasons. First, industrial
205 proteins expressed in plants can be synthesized in other systems and the alternatives are more
206 viable technically and economically. Secondly, several studies have shown that higher plants
207 accumulate waste products and heavy metals and these substances can irreversibly
208 compromise the quality of the proteins produced in these plants [58-60]. Thirdly, due to the
209 seasonal growth of most plants, adequate and constant supply of recombinant proteins is
210 always constrained in between the seasons. Lastly, there are possible contamination of plant
211 proteins with pesticides, herbicides and plant metabolites as well varying growth conditions
212 due to differences in soil quality and climate [47].

213 Other challenges result from the post translational modifications (PTMs) of proteins
214 expressed in plants. The PTMs such as glycosylation and proteolytic processing may
215 influence the stability, function and biological activity of plant therapeutic proteins [20, 43].
216 Studies have confirmed that defects in a number of genes in the glycosylation pathway cause
217 congenital disorders with serious medical consequences [61]. However variation in the
218 glycosylation profiles of specific therapeutic proteins have been recognized in diagnostics as
219 disease markers [62]. Plant protein glycosylation can introduce heterogeneity through the
220 generation of different key oligosaccharides and variable addition of outer arm sugars as the
221 glycoprotein transits the Golgi [63]. The nature and presence of these oligosaccharides may
222 affect the glycoprotein folding, stability, trafficking and immunogenicity including its
223 primary function [64]. The modifications usually range from simple chemical changes, such
224 as the addition of small phosphate or acetate functional groups to complex changes that are
225 enormous in size and mass exceeding the original protein [65, 66]. The PTMs are strictly
226 regulated, specific and may be due to developmental stage, location or by certain biotic or
227 abiotic factors, mainly by stress and disease state of a plant [67]. The cleavage of a signal
228 peptide or glycosylation is permanent changes of a protein structure compared to others like
229 phosphorylation that are rapidly and easily reversed [43].

5 Conclusion

The use of plants as expression systems has emerged as a promising system for the expression, production and manufacture of proteins of high value to health, food and industrial sectors. There are more advantages of plants compared to other traditional bioreactors. Typically, plants systems are superior in terms of safety, storage, complexity, cost and distribution relative to microbes, animals and insect cell cultures. Glycosylation, targeting, compartmentalization and natural storage stability in certain organs continue to make plants the preferred choice for production of proteins. Production of proteins in transgenic plants for commercial shows a great potential due to the potential economic benefits. Addressing challenges such as expression of proteins that are structurally and functionally similar to their native sources will make the plants ideal system for commercial production of recombinant proteins. It is vital to develop plant protein purifications systems that are safe and cost effective to meet the industrial need. The identification of the right candidate genes, market need and efficient production systems will build a strong link between plant protein screening and commercial utilization.

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