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Review article

Principles and Applications of Recombinant Protein Production in Plants

Bitkilerde Rekombinant Protein Üretimi Prensipleri ve Uygulamaları

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Abstract

Recombinant protein production has become a growing sector all around the world such as in therapeutic applications, pharmaceutical, agriculture. Although, several systems for protein expression have been developed, there is an increasing requirement for efficient methods of large-scale production. Therefore, recombinant proteins are produced by various ways to generate large quantities for commercial and research applications in a different host such as in bacteria, yeasts, insects, and mammalian culture. Among these, plant systems are mostly preferred since they have a developed eukaryotic system. Also, the expression of recombinant proteins in plants and plant cells has been promoted as an alternative cost-effective production platform. In this review we described, challenges and advantages of plants as expression systems for proteins and discussed unique advantages of producing proteins recombinantly in different plants; tobacco, rice, and maize.

Keywords: Recombinant protein, protein expression, plants, tobacco, rice and maize.

Özet

Rekombinant protein üretimi; terapötik uygulamalar, ilaç, tarım vb alanlarda dahil olmak üzere tüm dünyada büyüyen bir sektör haline gelmiştir. Protein ekspresyonu için çeşitli sistemler geliştirilmesine rağmen, büyük ölçekli üretim için etkin yöntemlere ihtiyaç duyulmaktadır. Dolayısıyla; rekombinant proteinler, ticari ve araştırma uygulamalarında kullanılmak üzere bakteri, mantar, böcek ve memeli gibi farklı sistemler aracılığıyla üretilmektedir. Bunlar arasında, bitki sistemleri sahip oldukları gelişmiş ökaryotik yapılardan dolayı en çok tercih edilen sistemlerden biridir. Ayrıca bitkiler ve bitki hücrelerinde rekombinant protein ekspresyonu, maliyet-etkin üretim platformu olarak desteklenmektedir.

Bu derlemede, proteinler için ekspresyon sistemleri olarak bitkilerin kullanılmasının zorlukları ve avantajları tanımlanmış ve farklı bitki türleri, tütün, pirinç ve mısır, kullanılarak rekombinat protein üretiminin avantajları tartışılmıştır.

Anahtar Kelimeler: Rekombinant protein, protein ekspresyonu, bitkiler, tütün, pirinç ve mısır.

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INTRODUCTION

Recombinant DNA (rDNA) is a DNA molecule that is produced by combining genetic materials obtained from different species through genetic engineering methods and is not otherwise normally found in nature. The rDNA technology is involved cutting and joining of DNA fragments from different sources and cloning in a host cell that can express so called recombinant proteins (James, 2007).

The production of recombinant proteins has become a main goal in biotechnology industries since structural and regulative functions of proteins are essential for life. The function of a protein is determined by the number and sequence of amino acids that shape the three-dimensional structure of the resulting polypeptide by folding. This process generates molecules with diverse and highly complex structure that can be used for many distinct applications. The use of therapeutic proteins for wellness and treatment have been approved in the United States and Europe for decades, account for almost a third of all pharmaceutical products in world (Walsh, 2018). Recombinant proteins are also frequently used in many applications such as the production of the textiles, chemicals and processing of food and feed. On the other hand; many recombinant proteins are routinely used in diagnostics or as research reagents. Consequently, demand for recombinant proteins is constantly increasing, with a market worth US$ 1.654 billion in 2017 forecast to reach US$ 2.850 billion by 2022 (Markets and Markets, 2017). Nearly half of this market is made up of therapeutic proteins (e.g., antibodies, vaccines, enzymes, cytokines, and growth factors) followed by industrial proteins (e.g., technical enzymes) and research reagents (e.g., antibodies for protein detection and purification) (Markets and Markets, 2017). The rapid growth on the market has been stimulated by recent developments in the technology of recombinant protein production, such as the identification of the expression system, of the desired protein and vector designing is essential to the manufacturing of recombinant protein and the development of efficient protein extraction and purification methods.

The recombinant proteins are also produced for basic research purposes to study the structure and function.

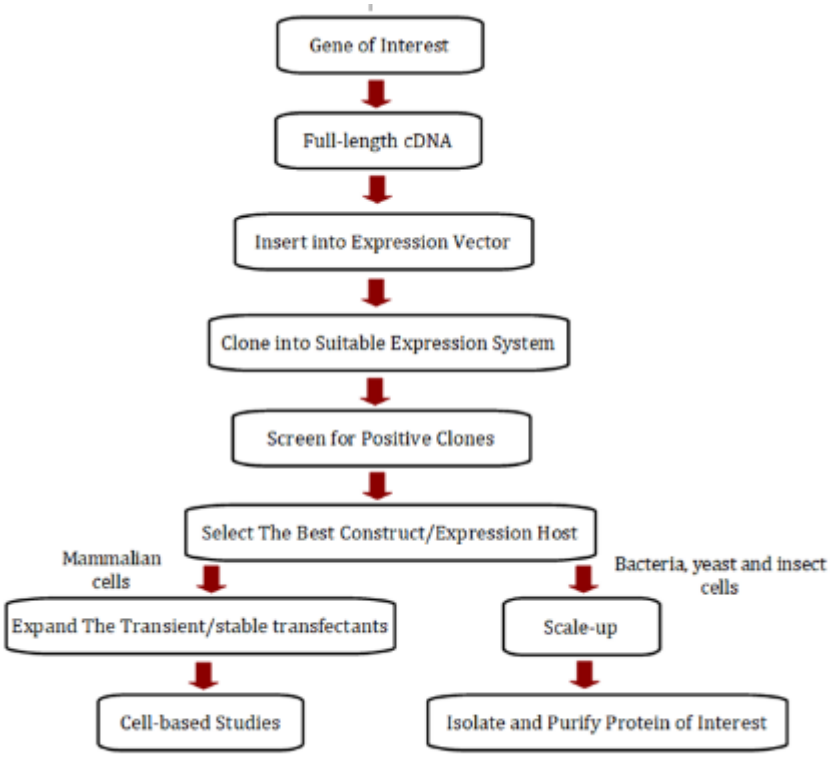

Figure 1. General Mechanism of Recombinant Protein Production

Today, various biological systems including bacteria, yeast, insect cells, fungi, transgenic animals and plants, as well as mammalian cell cultures are often used in production of recombinant proteins. Even though these hosts have different advantages and disadvantages, the ability to produce high-quality recombinant protein depends on high yields and low costs (Rasala et al., 2010; Schillberg et al., 2019).

Currently, the recombinant proteins have been produced in prokaryotic cells (mainly in *Escherichia coli*) although highly successful eukaryotic expression systems also exist. The key success in bacterial systems are easy handling, low costs, high yield and the opportunity to optimize the processes through affordable scaling-up processes (Schmidt, 2004).

However, the prokaryotic system has some limitations. The post-translational modifications such as glycosylation or disulfide bond formation, do not completed in bacteria. Additionally; many proteins cannot be obtained in an active and soluble form from bacteria cells. In some cases, the incorrectly folded proteins are formed the inclusion bodies that hinders protein purification (Potvin and Zhang, 2010).

Yeasts are highly preferred as recombinant protein production systems since expression level of proteins is relatively higher comparing with other systems. The first studies to produce recombinant proteins focused on the 'baker's yeast' *Saccharomyces cerevisiae*, which has become a well-understood model organism (Wildt and Gerngross, 2005). However; the recombinant proteins produced in yeast are

usually hyperglycosylated that alters immunogenic epitopes, and decreased in vivo half-life, that results in the loss of the therapeutic activity of the proteins (Potvin and Zhang, 2010).

Insect cells are also considered as an important alternative expression system for many biotechnological applications since they are more tolerant for the osmolarity changes and by-product accumulation. Although, the baculovirus-infected insect cells produce higher concentration of recombinant proteins, these systems have two disadvantages; they require complex nutrients and suffer from endogenous proteases, released naturally due to lytic cycles of virus that dramatically lower product yields (Ikonomou et al., 2003).

Although fungal expression systems are usually preferred for producing fungal enzymes, the intrinsic elevated concentration of proteases and the technical constraints of scale-up have restricted this mechanism to a few objectives as an expression (Punt et al., 2002).

Mammalian cell cultures are favored to produce the complex proteins since they have mechanisms for posttranslational modifications essential for the correct function of therapeutic proteins. However, the mammalian cell culture systems are very expensive to develop and maintain since they required complex nutrients and oxygen circulation. Also, these systems might be easily affected by waste accumulation, microbial contaminations, and sensitivity to shear stress (Wurm, 2004; Zhang vd., 2010).

Table 1. Comparison of Different Recombinant Protein Expression Systems (Potvin and Zhang, 2010).

System	**System Characteristic**							
	Molecular				**Operational**			
	Glycosylation	**Gene Size**	**Sensitivity to shear stress**	**Recombinant product yield**	**Production time**	**Cost of cultivation**	**Scale-up costs**	**Cost for storage**
Bacteria	None	Unknown	Medium	Medium	Short	Medium	High	Low (-20 ºC)
Yeast	Incorrect	Unknown	Medium	High	Medium	Medium	High	Low (-20 oC)
Insect	Correct, but depends on strain and product	Limited	High	Medium to high	Long	High	High	High (liquid N2)
Mammalian cells	Correct	Limited	High	Medium to high	Long	High	High	High (liquid N2)
Plant cells	Correct	Unlimited	N/A	High	Long	Low	Very low	Low (room temperature)
Unicellular microalgae	Correct	Unlimited	Low	Generally low	Short	Very low	Low	Low (room temperature)

In recent years, the development of deconstructed virus-based vectors has enabled plants to become a feasible platform for recombinant protein production with several advantages comparing other platforms in terms of versatility, speed, cost, scalability and safety (Chen and Lai, 2015).

Plants are increasingly considered as a platform for recombinant protein production since they provide certain advantages that are not found in other systems (Specht et al., 2010). First of all, main advantage of the plant systems is the potential for a significant reduction in cost (Dove, 2002).

The plant-based recombinant proteins are not contaminated by viruses or prions that can infects humans (Chebolu and Daniell, 2010). Also, plant cells can fold the complicated human proteins correctly since they have chaperones which are not available in bacteria and yeasts (Franklin and Mayfield, 2004).

Plants can also perform glycosylation of the complicated human proteins. Moreover, the targeted manipulation plant's N-glycosylation system enables the production of proteins with mainly homogeneous human oligosaccharides (Bosch et al., 2013).

Interestingly, plants have specific tissues (seeds) that can store high purity recombinant proteins in various organs including tubers and seeds (Tian and Sun, 2011; Torrent et al., 2009).

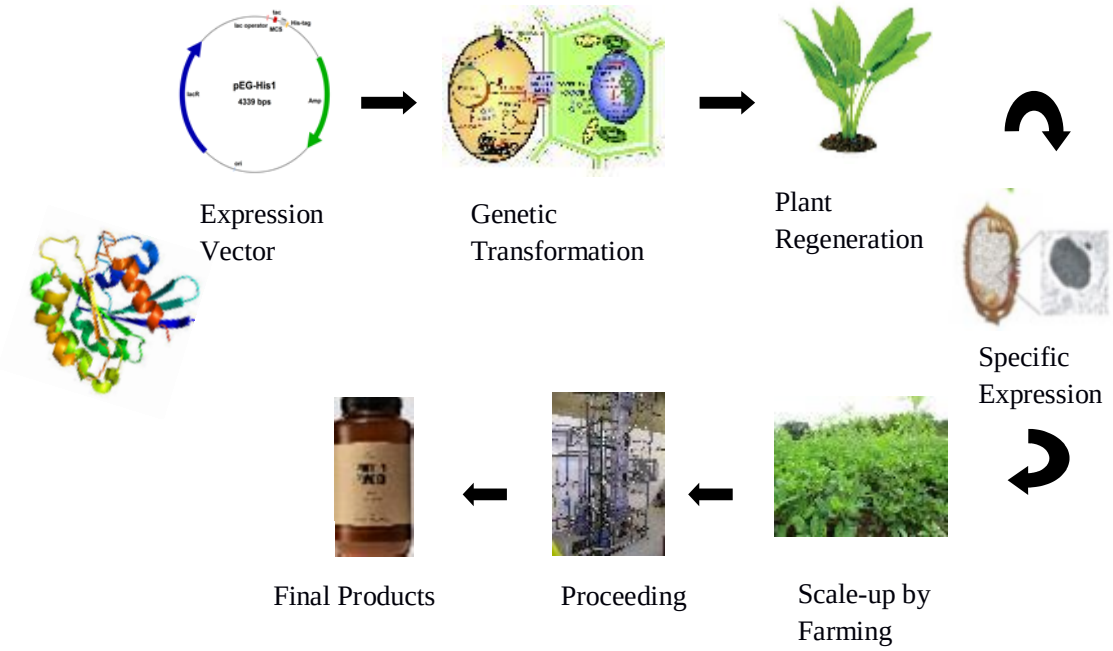

Figure 2. General Mechanism of Recombinant Protein Production in Plants

Despite the growing interest for plants in recombinant protein production, some limitations are also existing such as low level of expression, accumulation and incorrect post-translational modifications (Desai vd., 2010). For example; plant proteins lack the terminal residues of galactose and sialic acid frequently found in animals and have α-(1,3) fucose and β-(1,2) xylose which are absent in

mammalian systems (Malabadi et al., 2012). Also, some studies show that higher plants may accumulate heavy metals and waste products, so these products can irreversibly compromise the quality of produced in these plants (Kawaka and Ngetich, 2017).

The optimization of recombinant protein expression is also critical (Lau and Sun, 2009). To obtain a high expression level of recombinant protein in plants, constitutive promoters are required such as 35S promoter in dicotyledonous. Also, seed-specific promoters might be chosen to deposit the recombinant proteins in seeds, and polyubiquitin-1 promoters for monocotyledons (Christensen et al., 1992). Moreover, there is another factor which is the stability of transgene in plants (Lau and Sun, 2009). The codon optimization is a significant issue for recombinant protein production in plants since may cause problems during protein synthesis (Gustafsson et al., 2004).

Furthermore, the subcellular compartments of plant tissues such as chloroplast, mitochondria, and endoplasmic reticulum can be also considered to maintain of protein stability and accumulation efficiency (Hood et al., 1997).

Recombinant Protein Production in Tobacco

Nicotiana tabacum or cultivated tobacco is a member of flowering plants generally used in recombinant protein production due to the high soluble protein expression capability compared to other plant species, also can also be harvested 3-4 times in a year providing high efficiency of protein production per leaf biomass and seed (Conley, Zhu et al. 2011). Especially tobacco leaves are used as a fundamental part to reap the expressed proteins due to its plentiful manufacturing (Menkhaus, Bai et al. 2004).

The TMV-based expression vector is the most common application for the production of various therapeutic proteins in tobacco and BHV-1gD protein was produced by the TMV-30B vector, which is a type of TMV-based protein expression vector. It was shown that the protection of the natural host against viral challenge was induced with the plant extracts containing the gDC protein (Pérez Filgueira et al., 2003). Another example that; collagens are, also called body proteins, the most abundant form of proteins, which are present in all connective tissues of mammalians. Also, the use of a tobacco plant for the fully processed triple-helical molecules was appropriate. Therefore, the use of tobacco is the proper alternative source of collagen for producing good results without costing a lot in broad-range manufacturing (Ruggiero et al., 2000).

According to the study performed by Bailey et al., it was proposed that tobacco BY-2 suspension cells could be used for hydrophobin-assisted recombinant protein production. The hydrophobins (HFBs) are secreted proteins of fungi and have amphophilic properties, which demonstrate both hydrophilic and hydrophobic properties (Hakanpää, Szilvay et al. 2006). It was reported that the generation of protein bodies and effective purification of green fluorescent protein hydrophobin (GFB-HFBI) fusion was

achieved by aqueous two-phase separation (ATPS) and integration of HFB-fusion-technology in large-scale tobacco BY-2 suspension cell culture. So, they reported that the cell lysate captured the recombinant protein by surfactant-based ATPS and hydrophobin-fused GFP collected in endoplasmic reticulum derived protein bodies. Furthermore, it was demonstrated that a standard tank of bioreactors could be used for the propagation of BY-2 suspension cells (Reuter et al., 2014).

Recombinant Protein Production in Rice

Oryza sativa and *Oryzaglaberrima* are mainly developed rice species, which partly grow in the aquatic environment. Brown rice is an edible part of the rice which is also protected by a coat (Menkhaus, Bai et al. 2004). It was reported that the production of T-lymphocyte antigen 4-immunoglobulin (hCTLA41g) that competitive inhibitor in the CD28/B7 pathway, recombinantly was achieved by transgenic rice cell suspension cultures without disrupting the biological role of the protein It was successfully concluded that the recombinant hCTLA41g production could be performed by developing a trustworthy and effective protocol for the cryogenic storage in transgenic rice cells (Cho et al., 2007).

The studies of Wu et al. report the expression of human lysozyme (Hlys) in rice calli and cell in suspension cultures. The Hlys is one type of major human milk protein. It plays a role in the gastrointestinal tract of breast-field infants to the reduction of microbial infections. The study showed that after the removing of sugar from the culture medium, fully active rHlys were obtained by placing a synthetic, codon-optimized gene for Hlys under the control of a sugar repressible promoter (Huang et al., 2002).

Ning et al. showed that rice seeds could be used as a host for high scale expression of Oryza sativa recombinant human serum albumin (OsrHSA). The human serum albumin is a type of protein with globular, soluble, and unglycosylated monomeric properties. It has some functions that are initially as a produced transporter protein for steroids, thyroid hormones, fatty acids, and also plays a crucial role in extracellular fluid volume stabilization. After the experimental procedure, the study showed that OsrHSA was a congruent to plasma-derived HSA (pHSA) in the physical structure, functions, biochemical properties, and immunogenicity, which means that the using of rice endosperm is hopeful and harmless alternative approach for the high yield rHSA production (He et al., 2011).

Recombinant Protein Production in Maize

Maize is a kind of the earliest foundation for developing yield and quality from teosinte by selecting 5000 years ago. Its production reached approximately seven hundred million tons in 2005. Therefore, it is known as one of the universal main cereal crops that has many different versions that are used for the extractions of side products such as starch, oil, and ethyl alcohol. Furthermore, it is used as food and feed. These edible varieties have been accepted by *Generally Recognize as Safe* (*GRAS*) status

from the U.S Food and Drug Administration FDA. Moreover, the molecular farming of maize is a favorite base property that is well-established agricultural generation (Ramessar et al., 2008).

Hood et al., reported the production and purification of avidin from the chicken white part of the egg and *Escherichia coli* β-glucuronidase (GUS) from transgenic corn. The GUS has some properties that can be used as a marker for transgenic plant studies, homotetrameric hydrolase, and utilized as a research chemical by several chemical companies. On the other hand, avidin is a homotetrameric protein with a mass of 16.8 K/subunit and used initially as an identifier reagent. Moreover, avidin is present into avian that reptilian and amphibian egg white (Hood, Witcher et al. 1997). At the end of the procedure, study showed that the transgenic corn can be used as a bio-reactor for recombinant protein production and transgenic seed including avidin and GUS can be put away at 283 K for over a year and able to be taken care of at circumferential temperature for up to two weeks without lack of activity (Hood et al., 1997).

Peterson et al. concluded that the production of recombinant aprotinin protein by using a transgenic maize line with the demonstration of the biochemical and functional properties of this protein that same to its native counterpart and can be effectively recovered by transgenic maize seeds. Aprotinin is a serine protease inhibitor and single 58 amino acid polypeptide with a molecular weight of 6511 Da. The folding and biochemical activity has been documented about its structure. Aprotinin is also referred to as bovine pancreatic trypsin inhibitor that influences trypsin, chymotrypsin, plasmin, and kallikrein, which are known as serine proteases (Zhong et al., 1999).

Table 2. Examples of Recombinant Proteins

Recombinant Protein	**Origin**	**Host**	**Effect**	**References**
BHV-1 gD	Escherichia coli	Tobacco	Essential host preservation against viral threats.	(Pérez Filgueira et al., 2003)
Collogen	Animal Tissue	Tobacco	Manufactured as completely treated triple helical molecules, which concerned in utilization of biotechnology.	An, Kaplan et al. 2014)
Recombinant hCTLA41g	Mammalian Cell Culture	Rice	The cure of autoimmune illnesses hCTLA41g used due to its immunosuppressive impact.	(Cho et al., 2007)
Human lysozyme (Hlys)	Breast Milk	Rice	Development nourishment of baby food.	(Huang et al., 2002)
Oryzosative recombinant human serum albumin	Human Blood	Rice	Using rice seed bioreactor that is trustworthy to obtain recombinant HAS.	(He et al., 2011)
7Crp peptide	Composed of 7 dominant human T-cell epitopes	Rice	Usage for protect and productive tolerogen for immunotherapy	(Takaiwa et al., 2007)
β-glucoronidose (GUS)	Escherichia coli GUS gene	Maize	Transgenic corn can be used like a bioreactor.	(Hood et al., 1997)

Perspective and Conclusion

Plants as an expression system for recombinant protein production have proven to be an effective method of expressing, producing and producing high-value proteins for the health, food and industrial sectors. So, there are so many advantages of plants compared to other traditional expression systems.

In general terms, plants are better than microbial, animal and insect cell cultures concerning their safety, complexity, cost, and delivery. Therefore, they have the capacity to create distinctive glycoforms. So, plants are becoming acceptable for recombinant protein production for human therapeutics, vaccine antigens, industrial enzymes, and nutraceuticals.

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