

Advances in Genetic Engineering and Biotechnology for High-Yield Heterologous Protein Production in Crops

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Abstract

The evolving needs of health, industrial and agriculture sectors have spurred the use of recombinant technology to produce fusion proteins. Plants have been expression platform of choice due to large biomass production and ease of cultivation. The gene of interest can be inserted into plant expression sequence and be regulated as per need. Development of strategies is underway to improve fusion protein expression, quality and stability of expressed protein. Regulations regarding use and cultivation of transformed plant expression systems need to be made for safe and sustainable growth of heterologous protein production industry.

Keywords: genetic engineering, heterologous protein, crops, biotechnology

Introduction

Advancements in recombinant technology have opened up new avenues for enhancing crop varieties, such as developing disease-resistant and high-yielding plants. This same principle is now being utilized to produce foreign proteins in plants for molecular farming on a large scale. As a result, many transgenic plants have successfully been used to produce heterologous proteins. It is possible to create and manufacture proteins that do not occur naturally by designing and producing them in species that are not typically associated with them. Such proteins are called heterologous proteins and the process is referred as heterologous protein production.

This review will provide an overview of the genetic engineering and biotechnology methods used to improve crop yield and efficiency for heterologous protein production (Schmitz et al, 2020). We will discuss the different methods used to insert genes into plant genomes, plant-specific regulatory sequences for gene expression, protein isolation and purification methods, and strategies for enhancing protein expression and stability. Additionally, we will examine the regulatory considerations associated with the cultivation of genetically modified crops and the release of recombinant proteins.

Importance of crop improvement for heterologous protein production-

Heterologous proteins have been used in various fields like pharmaceuticals, synthetic production, agriculture, biomedical research, food production, bioengineering etc. (Ma *et al*, 2005; Paul & Ma, 2011; Stoger *et al*, 2014).) These and many other potential industries would be using these proteins in the times to come. Table 1 highlights some of the industries that already use heterologous protein products.

Table 1: Applications of Heterologous proteins produced in crops.

Application	Protein	Crop	Function	References
Pharmaceutical production	Human growth hormone	Corn	Stimulates growth and development in children	Ghag <i>et al</i> , 2021; Shanmugaraj <i>et al</i> , 2020; Juliane <i>et al</i> , 2013
	Monoclonal antibodies	Tobacco	Targets specific cancer cells for destruction	
	Vaccines	Rice	Stimulates the immune system to prevent infectious diseases	
Industrial production	Enzymes	Potato	Used in industrial processes such as brewing and paper production	Piscitelli <i>et al</i> , 2010
	Biodegradable plastics	Soybean	Used in the production of sustainable and environmentally friendly	

			products	
	Biofuels	Algae	Used as an alternative to traditional fossil fuels	
	Textile production	Flax	Used to produce materials with desirable properties such as softness and durability	
Agricultural production	Pest-resistant crops	Maize	Produces toxins that repel insect pests, reducing the need for chemical pesticides	Ali <i>et al</i> , 2020; Karavolias <i>et al</i> , 2021; Pitzschke, 2013
	Herbicide-resistant crops	Soybean	Resistant to herbicides, allowing for more efficient weed control	
	Nitrogen-fixing crops	Legumes	Converts atmospheric nitrogen into a form that can be used by plants, reducing the need for synthetic fertilizers	

Plants are indispensable expression platforms for fusion or heterologous proteins (Ghag *et al*, 2021). Thus, the potential uses in coming time necessitate improvement of crops. The efforts to improve crops are made to-

1. Achieve enhanced yield by increasing efficiency of protein expression.
2. Achieve stability of proteins during production and storage for commercial success of crops.
3. Improve resistance to stress conditions like drought, temperature or humidity.
4. Enhanced protein expression reduces the cost of production
5. The protein products obtained will be of more consistent quality, essentially required for pharmaceutical applications.
6. Easily harvest and process using simple inexpensive laboratory techniques.

Genetic Engineering for Heterologous Protein Production

The heterologous proteins can be produced in plants by recombinant technology. It involves identifying or synthesizing the gene sequences of interest followed by insertion into cloning and expression vectors. The expression vectors contain promoters for the desired protein expression in all cells of the plant, or inducible promoters that activate expression at specific stages, in specific tissues or organs, or in response to specific stimuli.

Methods for gene insertion into plant genomes

Agrobacterium tumefaciens is preferred biological vehicle to deliver genetic material for nuclear expression (Iyappan *et al*, 2019). It can carry large T plasmids with ability to transfer DNA into plant hosts, and its simple genome causes minimal rearrangements. This results in higher efficiency and low cost of production (Marillonnet *et al*, 2005). Additionally, viral vectors like tobacco mosaic virus can be used for DNA delivery in whole host plants, allowing for large-scale expression of genes of interest, such as those for therapeutic proteins like Hepatitis B surface antigen and single-chain antibodies. Other methods for DNA transfer include ultrasonication, liposome-mediated gene transfer, electroporation, and the particle bombardment gun (biolistic transformation) (Zhang & Wang, 2015). Each method has advantages and disadvantages, and different plants require different techniques for successful transformation. Overall, these techniques have enabled the non-pathogenic production of antivirals and other beneficial proteins in plants for safe human consumption.

Plant-specific regulatory sequences for gene expression

Plant-specific regulatory sequences are utilized for controlled expression of heterologous genes in plants. Once the gene is integrated into the plant genome, plant-specific regulatory sequences such as promoters, enhancers, and terminators can be used to express it (Schmitz *et al*, 2020). Plant-specific regulatory sequences are DNA sequences that regulate gene expression. They determine when and where a gene is expressed in a plant's development or in response to environmental stimuli.

Promoters are DNA sequences located upstream of a gene that initiate the transcription of the gene into RNA. Enhancers are regulatory sequences that enhance or increase the transcriptional activity of a promoter, while silencers decrease the transcriptional activity of a promoter. Other plant-specific regulatory sequences include cis-acting elements, which are short DNA sequences that interact with transcription factors, to regulate gene expression. These elements can be located in various regions of a gene, including promoters, introns, and untranslated regions (UTRs). Plant-specific regulatory sequences enable the creation of genetically modified plants with desirable traits, such as improved yield, resistance to pests or disease, or increased nutritional value (Schmitz *et al*, 2020). Researchers are enabled to precisely manipulate the levels and timing of gene expression to achieve the desired effect.

Protein isolation and purification methods

The common methods used for isolating and purifying proteins from the plant tissue include column chromatography, gel electrophoresis, and immune-precipitation (Wingfield, 2015).

Affinity chromatography: This technique uses a specific ligand, such as an antibody, to bind to and isolate the protein of interest from a complex mixture. The ligand is immobilized on a solid support and the mixture is passed through the support. The protein of interest binds to the ligand, while other proteins are washed away.

Ion exchange chromatography: This method separates proteins based on their net charge. The protein mixture is passed through a column containing a resin with charged functional groups. Proteins with a positive net charge bind to a negatively charged resin, while negatively charged proteins bind to a positively charged resin. The bound proteins can be eluted using a salt gradient.

Size exclusion chromatography: This method separates proteins based on their size and shape. A gel filtration column with a porous resin is used to separate the proteins by size. Larger proteins pass through the column more quickly than smaller ones, resulting in separation of the protein mixture.

Reverse-phase chromatography: This technique separates proteins on a hydrophobic stationary phase from hydrophilic proteins in the mixture.

Precipitation: This method involves adding a precipitating agent, such as ammonium sulfate, to the protein mixture to induce the proteins to aggregate and form a visible precipitate. The precipitate is then collected by centrifugation and washed to remove impurities.

Dialysis: This technique is used to remove salts and other small molecules from the protein solution with the help of dialysis membrane. The small molecules diffuse out while retaining the protein of interest.

These methods can be used individually or in combination to isolate and purify heterologous proteins from complex mixtures. The choice of method depends on the specific properties of the protein and the desired level of purity.

Choice of Crop

Heterologous proteins production involves using host organisms, such as plants, bacteria or yeast to produce a protein not naturally found in that organism (Feng *et al*, 2020). The choice of crop for heterologous protein production is critical, as different crops have different advantages and disadvantages in terms of protein production yield, protein quality, cost and scalability (Ghag *et al*, 2021).

Factors to consider when selecting a crop for heterologous protein production-

- 1. Expression System-** The limitation of prokaryotic systems to express human proteins necessitated need for eukaryotic expression systems. However the selected crop should have efficient expression system, strong promoter to drive protein expression and stability of protein in host crop (Feng *et al*, 2020). The presence of chaperons, post-translational modification machinery will influence the quality of the protein produced.
- 2. Protein of interest-** The choice of crop will depend on the protein of interest, as some proteins may be better expressed in certain crops. The crop should be capable of producing high quality protein that is correctly folded, post translationally modified and biologically active.
- 3. Ease of transformation-** The ease of transformation is an important consideration when selecting a crop for heterologous protein production.
- 4. Availability of genetic resources-** The availability of genetic resources, such as a complete genome sequence becomes crucial in the selection of a crop for heterologous protein production.
- 5. Commercial value-** The commercial value of the crop is another crucial factor to consider when selecting a crop for heterologous protein production (Morandini *et al*, 2011). The crop should be cost effective to grow and produce. The availability of seeds, timely maturity and ability for large scale production may affect the cost of target protein production.

Examples of crops commonly used for heterologous protein production

The selection of plants for protein production can affect expression of fusion proteins (Xu *et al*, 2011). Many crop plants like maize, tobacco, potato, tomato and soybean have been used for heterologous protein production. These plants provide large biomass and are easy to transform using *Agrobacterium* (Iyappan *et al*, 2019; Moustafa and Makhzoum, 2016). These can be easily engineered to produce wide range of proteins of clinical use e.g. Ebola virus vaccine production in tobacco, human growth hormone and erythropoietin production in maize (Gómez-Galera *et al*, 2007). The kernels of maize have been modified for this purpose. Overall maize and tobacco are valuable platform for production of various therapeutic proteins and other useful products.

Rice has also been a crop of choice as it serves a staple food for masses. It has been successfully transformed for production of including human serum albumin, lactoferrin, and α -amylase inhibitor. Soybean is another major crop, and its seeds have been engineered to produce therapeutic proteins such as monoclonal antibodies and biodegradable plastics (De Muynck *et al*, 2010). Additionally, soybean plants have been used to produce a protein that can be used as a vaccine against the porcine epidemic diarrhea virus (PEDV). Potato is a valuable food crop and has been genetically modified to produce vaccines for diseases such as hepatitis B and cholera. Overall, these crops have the potential to provide a cost-effective and safe method for producing a variety of therapeutic proteins for human and animal use. Each crop has its advantages and disadvantages, and the choice of crop should be based on a careful evaluation of these factors.

Strategies for Enhancing Protein Expression

The production of high yields of recombinant proteins in crops has its own challenges. Several strategies can be employed to improve protein expression including the following (Feng *et al*, 2020):

Strong Promoters- Promoters are regulatory sequences that control the expression of genes. Strong promoters can increase the expression of heterologous genes in plants, leading to higher yields of recombinant protein (Liu & Timko, 2020).

Codon Optimization- The codon usage of a gene can affect protein expression levels in plants. Codon optimization involves modifying the codon usage of a gene to match the codon usage of the host plant, resulting in higher levels of protein expression (Liu & Timko, 2020). The turnover of transgene product can be improved by increasing quantity of tRNA and rate of translation. Codon optimization can achieve 20-80 fold higher protein expression as against unmodified codons (Lico *et al*, 2008).

Strategies for enhancing stability of heterologous proteins produced

The extended duration in stability of fusion proteins can be accomplished by

Protease Inhibitors- Proteases degrade recombinant proteins in plant cells. Inhibiting protease activity improves protein stability and increases protein expression levels (Liu & Timko, 2020).

Fusion to Carrier Proteins- Carrier proteins may be used to apply with the protein of interest for better protein stability and protein expression levels.

Fusion to Ubiquitin- Ubiquitinylation of the protein of interest also improves protein expression levels and protein stability.

Subcellular Compartmentalization- Targeting the protein of interest to specific subcellular compartments can improve protein stability and reduce proteolytic degradation (Liu & Timko, 2020). Strong promoters can transcribe target sequences in some specific tissue.

Glycoengineering- Glycosylation is a post-translational modification that can affect protein stability and activity. Glycoengineering involves modifying the glycosylation patterns of a protein to improve its stability and activity. It also helps to produce humanised glycosylation pathways in plants (Liu & Timko, 2020).

Regulatory Considerations

The regulatory considerations for the production of heterologous proteins in plants are complex and depend on the intended use of the protein. For example, if the protein is intended for human consumption, it must meet strict safety and efficacy standards set by regulatory agencies such as the FDA. Additionally, regulatory approval may be required for the cultivation of genetically modified crops and the release of recombinant proteins into the environment.

The cultivation of genetically modified crops and the release of recombinant proteins are regulated by multiple government agencies like the American: Food and Drug Administration (FDA), the United States Department of Agriculture (USDA), and the Environmental Protection Agency (EPA). The FDA regulates the safety of genetically modified foods, including crops that have been genetically modified to produce recombinant proteins (Fischer & Emans, 2000). The FDA evaluates the safety of the proteins themselves, as well as any potential effects on the crops and the environment. The USDA is responsible for regulating the cultivation and distribution of genetically modified crops, the development and testing of these crops to ensure that they do not pose a threat to other crops or to the environment. The EPA is responsible for regulating the use of pesticides and other chemicals and evaluates the potential environmental impact of these crops and the chemicals used in their cultivation.

Proteins Recombinant proteins intended for human consumption must meet safety and efficacy standards before they can be approved for use. These standards are established by regulatory agencies and are designed to ensure the safety and efficacy of recombinant proteins. The cultivation of genetically modified crops and the release of recombinant proteins can adversely affect environment. These impacts are considered by regulatory authorities and mitigated to ensure the safety of the environment.

Future Directions

The potential of crop improvement for heterologous protein production need to be materialized to full extent. Regulatory hurdles, intellectual property concerns, and public perception may hinder the progress of research and development in this field (Sabalza *et al*, 2014). Advances in genetic engineering and biotechnology continue to improve the efficiency and yield of heterologous protein production in crops. Promising developments include the use of CRISPR/Cas9 gene editing and the development of novel plant-specific regulatory sequences (Schmitz *et al*, 2020; Yang *et al*, 2020; Lomonosoff & D'Aoust, 2016).

Conclusion

Crop improvement for heterologous protein production is a promising field of biotechnology with vast potential for the production of therapeutic proteins, vaccines, and industrial enzymes. Advances in genetic engineering and biotechnology have made it possible to

produce recombinant proteins in plants with high yield and efficiency. However, several challenges such as regulatory considerations, ethical concerns, and the need for sustainable agriculture must be addressed to realize the full potential of this technology.

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