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Review

Current Strategies to Improve Yield of Recombinant Protein Production in Rice Suspension Cells

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Abstract: A plant cell-based recombinant glucocerebrosidase was approved by the FDA in 2012 for the treatment of human inherited Gaucher disease, indicating that plant suspension cells have advantages in biosafety and a low production cost as a commercial pharmaceutical recombinant protein expression system. A low allergenic rice suspension cell-based recombinant protein expression system controlled by the *αAmy3/RAmy3D* promoter has been shown to result in relatively high protein yields in plant cell-based systems. Although several recombinant proteins have been produced in rice suspension cell-based systems, yields must be improved to compete with the current commercial protein expression systems. Different strategies were performed and showed successful improvements in recombinant protein yields in this rice system. The review updates and highlights strategies for potential improvements of the *αAmy3*-based rice suspension cell-based system.

Keywords: *αAmy3* promoter; recombinant protein; rice suspension cell; signal peptide

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1. Introduction

The production of valuable recombinant proteins in bacteria, yeasts, insect cells, mammalian cells and even plant cells by genetic engineering has become an important and common biotechnology in medical uses, with an estimated market value of approximately USD 400 billion by 2025 [1]. Each type of host cell has its own characteristics; thus, even the same recombinant proteins often show differences in biological activity and stability among various host cell protein expression systems. More than 50% of human proteins and 40% of commercial recombinant pharmaceutical proteins are glycoproteins; therefore, eukaryotic cells are often required as host cells to perform glycosylation for these recombinant proteins to have their best performances [2]. Although the current commercial biosimilars are mostly produced by Chinese hamster ovary (CHO) cells [3], the susceptibility to feedback inhibition of animal host cells limits the production of pharmaceutical recombinant proteins. In addition, production costs by expensive serum or required growth factors and biosafety concerns from common animal pathogens have pushed scientists to seek nonanimal-derived eukaryotic cell expression systems. Plant cells have advantages in protein post-modification, low production cost and high biosafety, and they are therefore applied to produce pharmaceutical recombinant proteins [4–6]. Many biopharmaceutical proteins, including antibodies, antigens and therapeutic proteins, have been successfully expressed in plant cells, and some are in clinical trials [7]; therefore, plant cell expression systems have attracted global attention.

Plant suspension cells are commonly cultured in relatively simple and inexpensive liquid medium compared to microbial and animal cells [1,4–7]. The recombinant protein can be fused to a signal peptide and then secreted into cell culture liquid medium. Secretory recombinant proteins can be obtained easily without breaking plant host cells, and purification from medium proteins is much easier than purification from cellular proteins. Recombinant protein expression platforms based on suspension cell culture have been

generated from several plant species, such as rice, tobacco and carrot. The first plant recombinant pharmaceutical protein, human beta-glucocerebrosidase for Gaucher disease patients, was produced from carrot suspension cells and approved by the FDA in 2012 [8]. This commercial case shows that the plant suspension cell expression system can fit the requirements of GMP and government regulation. It also proves that plant suspension cell expression systems have the potential to compete with the two major commercial protein expression systems, microbial *E. coli* and mammalian CHO cells. This review article focuses on the rice suspension culture cell production system and its current development in the production of recombinant proteins for medical applications.

2. Advantages of Plant Suspension Culture Cells

Among the current well-developed foreign protein expression platforms, plant cells have many advantages compared with microbial fermentation and animal and insect cell culture. Microbial systems perform limited post-translational modification for recombinant genes derived from eukaryotic cells. Recombinant proteins expressed in the *E. coli* system commonly have incorrect protein folding and form insoluble inclusion bodies. These inclusion bodies require extra solubilization and refolding steps to allow recombinant proteins to fold into the correct structure and therefore increase the protein purification cost [9,10]. In addition, the cost of recombinant protein production is further increased to completely remove possible endotoxins produced during the bacterial culture process. On the other hand, it is more expensive to produce recombinant proteins in mammalian and insect cells than in plant cells because of artificial culture medium supplemented with a variety of expensive growth factors and cytokines to meet the needs of host cell growth, coupled with cell sensitivity to mechanical stress in the bioreactor [1,4–7].

Well-established plant cell culture technology has been applied for the production of valuable metabolites, such as artemisinin, chlorogenic acid and ginsenosides [11–14]. Simons et al. were the first to use potato and tobacco cells to produce medically valuable recombinant human serum albumin in liquid culture medium [15]. Plant cells cultivated in a sterile cell culture environment have more advantages than plants grown in greenhouses or fields. For example, agricultural fertilizers and pesticides are not used in the plant suspension cell culture expression system, and they therefore do not cause environmental pollution. There is no potential transgene contamination in plant suspension cells. In addition, the proliferation rate of plant suspension cells is high as compared to solid cultured cells, and the production time required to obtain recombinant protein is shorter than that of transgenic plants. Compared with the whole plant system, the plant suspension cell system produces recombinant proteins under a controlled aseptic culture environment and therefore improves the biological safety of recombinant proteins [16,17].

Purification of recombinant proteins remains the major cost of commercial recombinant protein products; thus, protein purification is an expensive and challenging step in the biotechnology industry [18,19]. Plant suspension cells can easily reduce the cost of recombinant protein purification by fusing the signal peptide sequence with the target gene, and the recombinant protein can be directly secreted into the extracellular culture medium. The strategy simplifies the process of recombinant protein purification by avoiding interference from a large amount of intracellular proteins. Therefore, using plant suspension cells to produce valuable recombinant proteins is a good, economical, safe and simple strategy, with the following advantages: (1) plant cells are eukaryotic, similar to animal cells, and can perform complex post-translational modification; (2) recombinant proteins produced by plants can be expressed in particular subcellular organelles, such as the endoplasmic reticulum, chloroplasts and vacuole [20,21]; and (3) the recombinant human proteins produced by plants can avoid cross-contamination of human or animal pathogens and have less interference from other family members, which share similar protein structures, to make protein purification easy and safe. Since the first successful case was reported in 1990, recombinant proteins, including medical vaccines, antibodies, cytokines, other protein

drugs, industrial enzymes and food additives, have been successfully expressed in plant cells [9,22–26].

3. Rice Suspension Cell Recombinant Protein Expression System

Tobacco and rice cells are commonly used in suspension culture to express recombinant proteins. In general, the constitutively active cauliflower mosaic virus CaMV 35S promoter is used to produce foreign proteins in tobacco cells. The yield of recombinant human antibody in tobacco cells in 200 L bioreactor was approximately 20 mg/L [27], and tobacco cells are known for the production of toxic alkaloids and proteolytic enzymes. On the other hand, the recombinant protein production platform of rice suspension cells generally has a protein yield more than 10 times that of tobacco cells [9]. Rice suspension cell protein expression systems have been established on a large scale to produce various recombinant proteins in several biotech companies, such as Natural Bio-Materials at Korea [28]. Several valuable pharmaceutical recombinant proteins produced from rice cells are under clinical trials, such as *Vibrio cholera* CTB (cholera toxin B subunit) for cholera [29]. Various growth factors, enzymes, interferons and antibodies have been successfully produced in rice cell suspension culture systems [22,30–33].

The recombinant protein expression system of rice suspension cells often uses a transgenic cell line containing the inducible *αAmy3* (also called *RAmy3D*) promoter and its signal peptide to secrete the recombinant protein into the culture medium under sugar starvation conditions [34,35]. Diagrammatic representation of the rice suspension cell expression system and process for various recombinant protein production under the control of *αAmy3* promoter and its signal peptide is shown in Figure 1. Briefly, a gene of interest, which may be modified with codon optimized sequence in rice, is cloned downstream of the *αAmy3* promoter-signal peptide sequence either by Gateway or restriction enzyme cloning technology. The high yield of stable transgenic rice cell lines can be screened after an expression cassette is transferred into the rice nucleus by Agrobacterium- or particle bombardment-mediated transformation. *αAmy3* is a member of the α-amylase gene family, and rice contains 10 α-amylase genes. When cereal seeds are in the germination stage, α-amylase genes in embryos and aleurone cells begin to express and secrete α-amylase into the endosperm to break down starch into small molecular sugars by cutting the α-1,4 glucose linkage in amylose and amylopectin molecules. The main α-amylase genes expressed in rice suspension cells are *αAmy3*, *αAmy7/RAmy1A* and *αAmy8/RAmy3E*, and their promoters are regulated by gibberellic acid, abscisic acid and carbohydrates. *αAmy3* has the highest expression level induced by glucose deficiency among the whole rice α-amylase gene family in rice suspension cells [36], and the expression level can be continuously enhanced for up to 48 h [37]. The signal peptide of αAmy3 can be applied to allow the fusion of recombinant proteins secreted into the extracellular medium to simplify the protein purification steps.

The rice suspension cell expression system is divided into two cell culture stages: the cell proliferation stage and the recombinant protein production stage (Figure 1). During the cell proliferation stage, the rice suspension cells are cultured in a liquid medium containing a carbon source, such as sucrose. Rice cells replicate and proliferate, and highly increase the number of rice suspension cells; then, the cell culture medium is replaced with a medium lacking a carbon source to activate the *αAmy3* promoter, and the recombinant protein is produced and secreted via the default pathway of the αAmy3 signal peptide into the liquid medium.

The advantages of this rice expression system are as follows. (1) An easy tissue culture procedure is enough to establish homogeneous and rapidly growing rice suspension cultured cells. Rice suspension cultured cells have a doubling time of about 2 days [38]. (2) The use of a strong inducible *αAmy3* promoter can be easily activated by the absence of sugar in the cell culture medium to express recombinant proteins. (3) The application of secretory signal peptides can be utilized to transport recombinant proteins from the cytosol into the culture medium. After simple collection of the cell culture liquid medium, the

recombinant proteins can be purified without breaking the cells. Continuous production of recombinant proteins can be carried out without sacrificing rice host cells. Rice cells can be reused for recombinant protein production, and the sugar feeding and starvation cycle also saves time for cell proliferation.

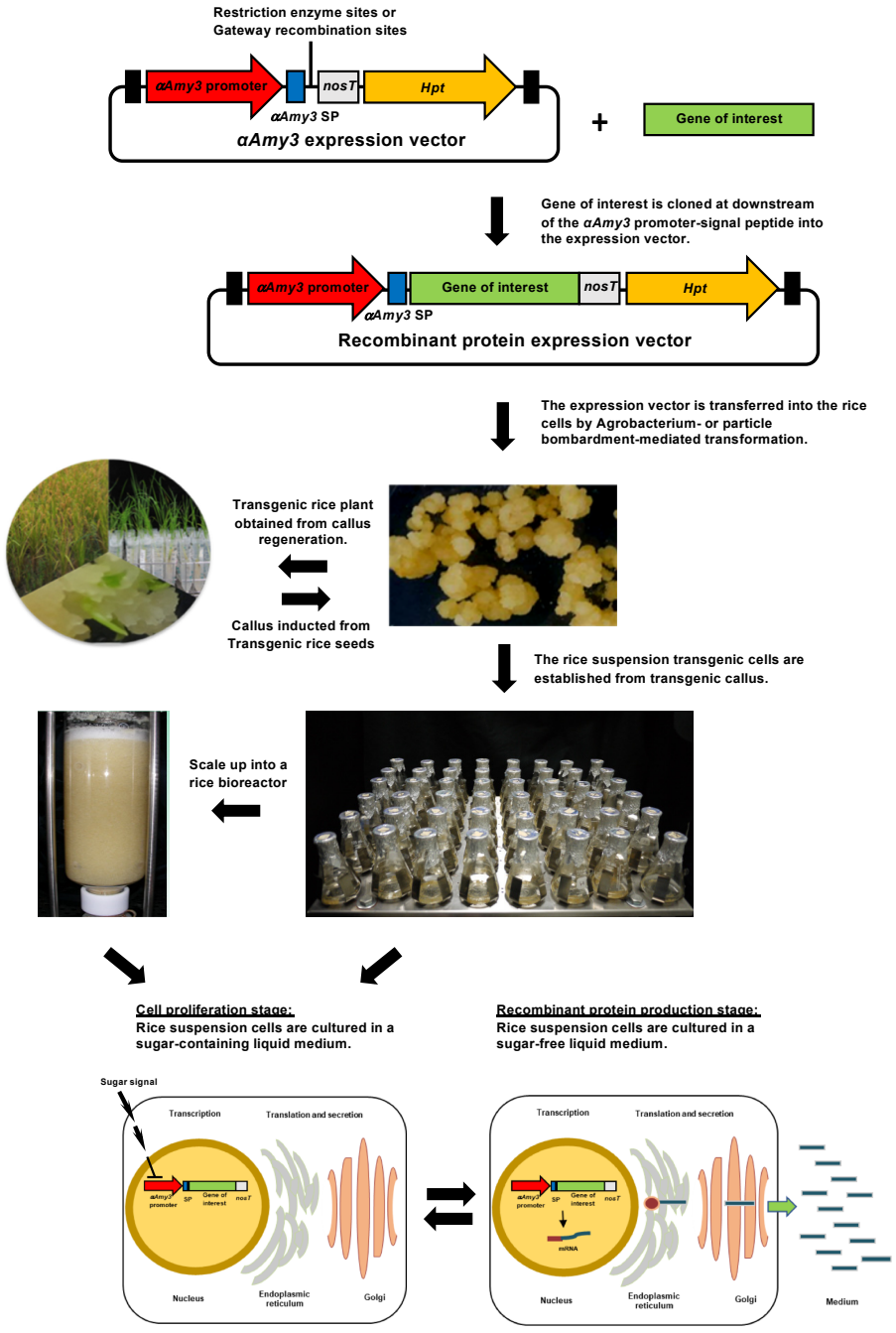

Figure 1. Schematic diagram of the *αAmy3* promoter-signal peptide-based recombinant protein production system. The expression vector contained the rice *αAmy3* promoter-signal peptide sequence, *nos* terminator (*nos*T), and the hygromycin selection marker (*Hpt*). The *αAmy3* promoter-signal peptide-gene of interest expression cassette is transferred into rice nucleus. The recombinant proteins are secreted into sugar-free liquid medium from rice suspension cells under recombinant protein production stage.

4. Approaches Applied to Enhance the Performance of the Rice Suspension Cell Recombinant Protein Expression System

Although plant protein expression systems avoid human pathogenic bacterium contamination and therefore have the advantage of high biosafety, plant protein expression systems are required to improve the yield of exogenous recombinant protein to compete with the current commercial protein expression systems. The highest recombinant protein production of rice cell bioreactor grades reported thus far is human α1 antitrypsin. The production of this recombinant antitrypsin was driven by the rice *αAmy3* promoter fused with its signal peptide, and the exogenous protein secreted into the culture medium reached 247 mg/L [39]. However, the two major commercial recombinant protein expression systems, CHO cells and *E. coli*, have yields that reach the g/L level on average so far. The *αAmy3* promoter has been widely used in sugar-regulated transgene expression in rice suspension cultured cells [9], several strategies have been reported to enhance the recombinant protein yield of the rice *αAmy3* promoter and signal peptide-based rice suspension cell expression platform and are briefly described as follows:

4.1. Regulation of Gene Expression in Rice

The promoter is key to regulating gene expression, and it is the most important factor affecting protein yield in the recombinant protein expression system. The promoters used in recombinant protein expression are roughly divided into constitutive expression promoters and inducible promoters. The use of constitutive promoters can routinely express recombinant protein, but it may lead to abnormal growth of host cells. The advantage of inducible promoters is that they only start under induction conditions, and they can thus avoid interference with normal host cell growth. Therefore, inducible promoters are better at driving the best performance of recombinant proteins in recombinant protein expression systems, including rice suspension cell systems.

During the seed germination stage of cereals, such as rice, corn, wheat and barley, α-amylase gene expression is induced with other hydrolytic enzymes to breakdown starch stored in the endosperm into smaller molecular sugars to provide a carbon source for seedlings. The rice α-amylase gene family comprises at least 10 gene members, among which *αAmy7*, *αAmy3* and *αAmy8* have the highest expression levels during seed germination [36,40,41]. Plant hormones, including gibberellins and ABA, and carbohydrates can regulate the expression of the α-amylase gene; for example, gibberellins promote the expression of *αAmy7* and *αAmy8*, but carbohydrates inhibit the expression of *αAmy3* and *αAmy8* [42]. In rice cell suspension culture, carbohydrates are the main factors regulating the expression of α-amylase genes. In the sugar-containing environment of rice suspension cell culture, levels of α-amylase mRNA decrease. When rice suspension cells are in a sugar-deficient period, the mRNA levels of α-amylase genes increase, among which the accumulation of *αAmy3* mRNA is the most apparent [34], and the *αAmy3* promoter is an important and key regulatory step for expression of the *αAmy3* gene induced by glucose deficiency [43]. Due to the inducibility and high expression level of the *αAmy3* promoter, it is widely used in the rice exogenous protein expression system to express a variety of different foreign proteins [9].

Genetic engineering for rice cells was carried out to improve the *αAmy3* promoter-based recombinant production system. Knockdown of endogenous *αAmy3* almost doubled the yield of recombinant protein production in transgenic rice cells [44]. Manipulation of transcription factors is another strategy to enhance *αAmy3* promoter activity. Rice α-amylase genes contain a TATCCA sugar response element at the promoter region [43]. The OsMYBS2 transcription factor was able to reduce *αAmy3* promoter activity by competing with the OsMYBS1 activator for this binding TATCCA sugar response element [45]. Reduction of *OsMybS2* expression by RNA interference (RNAi) technology increased the yield around 5 times of exogenous mGM-CSF in the *αAmy3* promoter-based rice recombinant protein expression system [46].

Recently, CRISPR/Cas9 knock-in technology was successfully developed for a recombinant protein production under the control of the *αAmy3* endogenous (native) promoter and signal peptide in rice suspension cells [47]. In this system, an artificial 3′ splice site was added at 5′ end of the recombinant gene and inserted directly into intron 1 of *αAmy3*. The intron was spliced out of the *αAmy3SP*-recombinant gene pre-mRNA to generate a mature mRNA, and then the in-frame αAmy3SP-recombinant fusion protein was translated. The CRISPR/Cas9 knock-in transgenic rice cell lines showed that recombinant GFP expression was regulated by sugar starvation induced *αAmy3* endogenous promoter, and the GFP protein was secreted into the culture medium by the αAmy3 signal peptide [47]. The activity of the endogenous *αAmy3* promoter is strongly induced by glucose deficiency [34], and this CRISPR gene editing technology is expected to increase the transcription of the recombinant gene by insertion downstream of the endogenous *αAmy3* promoter in rice suspension cells. However, conventional methods of recombinant gene expression in transgenic rice, using Agrobacterium- or particle bombardment-mediated transformation, an expression cassette is inserted at random locations in the rice genome with various copy numbers either. This results in various expression levels of the transgene among independent transgenic lines, and high-expression lines are required to screen from large numbers of transgenic lines. The modified CRISPR/Cas9 knock-in approach generates a precisely insertion at downstream of *αAmy3* promoter and signal peptide and therefore provides an efficient way to have a consistently high expression level of recombinant genes.

4.2. Screening of the Best Rice Signal Peptide

The best way to avoid sacrificing rice host cells is to directly secrete the recombinant protein into the liquid culture medium and then collect the target recombinant protein from the medium. Secretory recombinant proteins greatly reduce purification steps for protein collection and can avoid protein extraction to reduce high production costs [48]. Two strategies are generally applied to secrete the recombinant protein outside the plant cells; one is to use the secretory signal peptide from the recombinant protein. The protein secretion mechanisms of eukaryotic cells are basically similar, and the same signal peptide from one species can therefore be used in another species, such as human signal peptides used in yeast, animal and plant cells. The production of antibodies, interferon and cell growth hormone by tobacco suspension culture cells used the original signal peptide derived from recombinant proteins [49–51]. The advantage of this strategy is to simplify the construction procedure of the expression vector, but it is not suitable for recombinant proteins that are not originally secreted outside the cell. Another strategy is to fuse the recombinant protein with a secretory signal peptide derived from plant cells to bring the recombinant protein out of the plant host cells. A selected secretion signal peptide from plant host cells was applied for construction, regardless of the original cellular localization of the recombinant protein. For example, plant signal peptides are used in tobacco hair roots [52] and rice suspension cell recombinant protein expression systems [53].

The protein secretion pathway is conserved in yeast, animals and plants; the N-terminal signal peptide is translated and cleaved by signal peptidase on the ER membrane, and proteins are further transported through the Golgi and then finally to the plasma membrane, extracellular space or vacuole [54]. Most secretory proteins contain signal peptides, which are short peptide fragments of approximately 20–40 amino acids at the N-terminal protein that direct protein secretion. There is no conserved sequence among signal peptides, and different signal peptides have different secretion efficiencies. To improve the secretion efficiency of recombinant proteins in rice expression systems, a GFP reporter was applied to evaluate the secretion efficiency of three signal peptides, and the rice 33KD signal peptide was shown to be more efficient than the αAmy3 signaling peptide in secreting GFP, regardless of sugar-containing or sugar-deficient medium [55]. Thus, high-efficiency secretory signal peptides, such as the 33KD signal peptide, can contribute to the yield of recombinant proteins in rice suspension cells.

4.3. Regulation of Proteolytic Enzymes in Rice Cells

Several reports have shown that recombinant proteins can be detected in the cellular protein extract but not in the medium, implying that secreted protease digestion causes substantial loss of recombinant proteins [56,57]. Genome-wide gene expression profiling of sugar-deficient rice cells through microarray analysis showed that several proteases were significantly upregulated when rice cells were sugar deficient and the *αAmy3* promoter was activated [58]. Among them, cysteine proteases have been indicated to have negative impacts on the production of recombinant proteins [59]. The elimination of protein degradation in sugar-deficient rice cells or cultured medium efficiently increases the yield of recombinant protein. Two strategies of reducing protease effects have been used to improve recombinant protein yield in the rice suspension cell protein expression system. Genetic engineering by silencing cysteine proteinase in transgenic rice cells increased 1.9 folds of the yield of recombinant human GM-CSF [59]. Knockout of a specific protease gene (or genes) created by the CRISPR gene editing approach will likely be applicable in this rice expression system in the near future. Alternatively, specific protease inhibitors could be used to reduce the activities of certain proteases. Two serine protease inhibitor families, Type I (PI-I), which only inhibits chymotrypsin, and Type II (PI-II), which can inhibit both chymotrypsin and trypsin, have been found to play important roles in plant metabolism [60]. A serine proteinase inhibitor II gene (PI-II) from tobacco was ectopically expressed in a rice cell suspension culture and reduced protease activity, in turn increasing the accumulation of recombinant proteins [61]. Strategies to ectopically express protease inhibitors have been applied in other plant recombinant expression systems. Therefore, it is a useful method to protect recombinant proteins from extracellular proteolytic attack. Several secreted proteases have been found in sugar-free rice-cell-cultured medium. Isolation and manipulation of specific rice proteases, either through knockout or coexpression specific protease inhibitors, are worth studying and can be applied to increase recombinant protein levels in a sugar-free cell culture medium.

4.4. Optimization of the Culture Medium

Rice suspension cells proliferate in a sugar-containing culture medium, and cells sense sugar signals during the cell growth period and block the activity of the *αAmy3* promoter. Only when the rice cells are transferred into sugar-deficient medium or consume the sugar from the culture medium completely does the *αAmy3* promoter begin to express the downstream recombinant gene. If the recombinant proteins are harmful to rice host cells in this inducible *αAmy3* promoter-based expression system, the transgenic rice cells can remain well amplified in normal sugar-containing medium without the influence of cell activity. However, the *αAmy3* promoter is activated when rice cells run out of their carbon source, and this sugar starvation condition causes the cell activity to drop and results in a reduction in recombinant protein yield. Sugar is the carbon source provided for rice suspension cultured cells. One glucose molecule can be converted into two molecules of pyruvate during glycolysis. Pyruvate can enter directly into mitochondria, and the breakdown of pyruvate generates NADH molecules through the tricarboxylic acid (TCA) cycle to maintain cell viability [62]. Ho et al. (2001) found that pyruvate can increase the activity of the αAmy3 promoter in glucose-deficient rice cells [37], implying that rice cells can obtain pyruvate as an energy source and still sense sugar starvation signals via the hexokinase-mediated sugar sensing pathway to activate the *αAmy3* promoter. Masaaki et al. (2011) further proved that supplementation with pyruvate enhanced the activity of the *αAmy3* promoter to express recombinant human α1-antitrypsin in rice cells in a sugar-free environment [63]. Although Kim et al. (2014) claimed that pyruvate did not significantly increase the yield of either recombinant granulocyte-macrophage colony stimulation factor (GM-CSF) or trypsin under the control of the *αAmy3* promoter, adding intermediates of the TCA cycle, such as succinic acid, fumaric acid and malic acid, to the sugar-free medium could enhance the yield of recombinant trypsin [64]. Therefore, supplementation with specific carbon resources that avoid hexokinase-mediated sugar sensing pathways, such

as intermediates of the TCA cycle, can offer cell energy requirements to maintain cell viability and enhance the recombinant protein yield of *αAmy3* promoter-based expression systems. Increasing the cell activity in a sugar-free environment is clearly an important strategy to enhance the production yield of recombinant protein. Adding a small amount of glucose (0.5 mM) before the induction of recombinant protein production can also enhance rice cell activity, thereby increasing the yield of recombinant human α1-antitrypsin protein two-fold in a sugar-free environment [65]. In addition to providing an altered carbon source, the addition of amino acids such as glutamine, aspartic acid, arginine and glycine to glucose-deficient culture medium during the production phase can also increase the yield of recombinant protein by 1.2 times in an *αAmy3* promoter-based expression system [66]. Amino acids can be oxidized as an energy source to provide cell activity for protein synthesis under sugar-free conditions [67,68]. Moreover, since proteolytic enzymes are produced to degrade target proteins as energy resources in a sugar-deficient state [37], the added amino acids are the products of protein degradation and therefore can inhibit the production of proteolytic enzymes by the feedback regulation mechanism, which benefits the accumulation of recombinant protein in the *αAmy3* promoter-based expression system. Plants accumulate proline under stress, and proline increases the stability of membranes and the cell wall [69]. The exogenous application of proline increased rice cell growth, extended rice cell viability under sugar starvation, and significantly increased the 2-fold yield of recombinant human serum albumin driven by the *αAmy3* promoter in sugar-starved rice suspension cells [70].

4.5. Establishment of the Optimal Culture Procedure for Rice Suspension Cells

To activate the rice *αAmy3* promoter in rice cells, a sugar-free environment must be created either by replacement of the sugar-deficient medium or natural consumption of sugar by rice cells. Replacing the sugar-free medium increases the risk of contamination during the production process and also increases the cost of materials and labor. Rice cells can naturally consume carbon sources in the culture medium. Liu et al. (2015) optimized the cell culture protocol using a one-step strategy to naturally consume the carbon source in the culture medium without changing the medium and increasing the recombinant protein yield of human serum albumin by approximately 3-fold in the flask [71]. This method avoids the two-step sugar-free medium replacement, which could damage rice cells by glucose deficiency shock. This natural sugar deficiency method is more convenient in large-scale bioreactors and increases the yield of recombinant proteins [66,71]. For example, no media exchange method was applied to improve the *αAmy3* promoter-based expression system in the production of therapeutic rice recombinant butyrylcholinesterase (rrBChE) in a 40 L pilot-scale bioreactor [72]. Batch cultivation is the most common method for plant suspension culture cells to produce recombinant proteins, and no substances are added or removed during batch cultivation. The natural sugar consumption method allows rice cell growth and maintains cell viability to continuously produce recombinant proteins. The signal peptide is applied in this *αAmy3* promoter-based rice suspension culture cell production system to secrete the recombinant protein into the culture medium, and secreted recombinant proteins in the medium can be directly harvested without harming the cells. This method allows the same batch of cells to continuously produce the recombinant protein through the conversion of the sugar-containing or sugar-free medium. The production capacity of a batch of cells can reach up to 4–7 cycles [71,73]. The one-stage continuous production model was further extended to produce rrBChE recombinant protein in a pilot-scale bioreactor, which also maintained cell viability for 82 days and produced at least four cycles of recombinant proteins [72]. However, the cell concentration and turnover rate of suspension cells cultured in the bioreactor limit the reusability of rice suspension culture cells and the level of recombinant proteins produced. Immobilization of cells can improve the production cycle and the total cell production period. Moreover, the immobilized rice cells can be easily separated from the culture medium, and the secreted protein fraction

simplifies the later purification of recombinant protein. Recently, one case has been reported in which rice cells were immobilized for high-value recombinant rrBChE protein [74].

4.6. Optimization of Codon Usage

Codon optimization is frequently used to promote heterologous gene expression by accommodating the codon bias of host plants. For example, the yield of a recombinant stem cell factor (SCF) protein was increased 25- to 30-fold in tobacco BY-2 cells by codon optimization [75]. The preferred codon usage on protein expression was applied to enhance translational efficiency, previously. Recent evidence indicates that codon usage also correlates with mRNA levels [76–79]. Expression of human origin pharmaceutical recombinant genes in rice suspension cells are usually modified their coding sequences to match rice cellular activities and facilitate the yield of recombinant proteins. Rice-optimized codon usage was applied to synthesize human growth hormone (hGH) and human pepsinogen C (hPGC) gene in order to express in rice suspension cells under the control of the *αAmy3* promoter [33,80]. However, the efficacy of codon usage on recombinant protein production in rice suspension cells has not yet been demonstrated.

5. Conclusions and Perspectives

The current disadvantage for plant suspension cell expression systems via secretory pathways is the low production yield of recombinant proteins. Various efforts have been made to improve the *αAmy3* promoter-based rice suspension cell recombinant protein expression system, including an optimal cell culture medium recipe, a practical culture strategy for both cell growth and protein, high secretion efficiency options for signal peptides, and elimination of transcription repressor and protease activity, providing insights into the development of an optimal plant recombinant protein system. A combination of state-of-the-art strategies from present aspects will increase the yield of recombinant proteins to meet commercial standards. More research is required to optimize rice cell recombinant protein production processes to compete with the average yields produced by the CHO cell system. CRISPR gene editing technology is available for rice cell engineering and provides a great tool to further optimize *αAmy3* promoter activity through knockout of negative transcription factor genes, such as *OsMYBS2* and *OsMYBS3*. In addition, CRISPR knock-in technology has been established for the endogenous *αAmy3* promoter to produce recombinant proteins. Further comparison of transcriptional activity between the current use of the recombinant 1.1 kb *αAmy3* promoter and the native *αAmy3* promoter will define the improved levels of promoter activity. It is also important to achieve a better understanding of how to prevent protein degradation during the production stage of the rice suspension cell system. Adding proteinase inhibitors and blocking protease activity, including knocking out specific proteinase genes by CRSIPR as well as coexpression of protease inhibitors, will be useful for enhancing recombinant protein production in rice suspension cells.

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