# Production of Human Erythropoietin in Transgenic Canola Employing the Technology of Oleosin Fusion

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Human erythropoietin (EPO) is an endogenous cytokine that is responsible for the stimulation of the production of erythrocytes. Purified EPO is mainly used for treatment of anemia. Many conditions can result in unhealthy anemic levels; therefore, EPO can be used to care for many differing ailments. EPO was first successfully cloned in 1984. Since then, it has been produced in yeast, bacteria and insect cells. Additionally, it has been synthesized transgenically in plants. Mammalian cell systems have been shown to possess the highest efficiency for producing EPO, even though they require high cost; however, the appeal of using plant environments to transgenically produce proteins is extremely alluring due to its cost efficiency. One plant that has been shown valuable in its ability to transgenically accumulate proteins is Brassica napus, commonly known as canola. Canola has shown the capability of transgenically producing the hirudin protein through oleosin-fusion technology. This technology has been suggested to produce extremely stable recombinant proteins that exhibit their normally expected activity. Thus, here the employment of oleosin-fusion techniques is proposed as a template to produce recombinant EPO (rhEPO) in canola. If successful, this project will exhibit a new, stable and cost efficient method of producing the rhEPO that is so highly desired within the biopharmaceutical market.

## Introduction

The present project proposal is organized into five main sections. The bibliographic review is the first section. Within the bibliographic review, background information is given on the EPO, transgenic plant systems, canola and the oleosin-fusion technology. An understanding of the bibliographic review will make further reading of this proposal more easily grasped.

Following, the justifications and objectives of this project are explained. The general objectives as well as the specific objectives are provided in this section of the proposal. Additionally, a table relating to the specific time allotted for these objectives has been created.

How the objectives will physically be attained is clarified within the subsequent technical strategies division of the proposal.

The following division is laboratory infrastructure. This section addresses the laboratory, financial resources and materials needed to execute the project.

The final section is titled expected results and discussion. Within it, the expected results are given and a discussion explains this experiment's value and why the existence of patents on oleosin-fusion technology is not a deterrent from performing this proposal.

## Bibliographic Review

### *Erythropoietin*

The human erythropoietin (EPO) is a cytokine involved in the stimulation of erythrocyte

production in bone marrows. EPO production transpires in the kidneys of adults and liver of embryos. EPO has more influence on the flow regulation of these red blood cells than any other human hormone. Increased states of hypoxia, a result of physiological stimulation, have been shown to amplify the expression of EPO. These hypoxic states allow EPO to enter blood circulation. In addition to these deprived states of oxygen supply, EPO also enters into erythrocyte circulation when cobalt-chloride is simultaneously present in circulation [1-5].

Purified EPO is used to treat anemia, which is onset by several conditions including chronic renal failure, chemotherapy, frequent dialysis, surgery, the acquired immunodeficiency syndrome, rheumatoid arthritis associated with chronic anemia, or cell transplants including those of the kidney, bone marrow, and stem cells [4-10].

EPO displays strong tissue-protective behavior and has demonstrated protection towards the spinal cord, retina, brain, heart and kidneys. Its tissue-protective actions have been connected to several cytoprotectant pathways that are active during tissue injury or disease. EPO can be applied therapeutically to other conditions not associated with anemia. These conditions include autoimmune disorder treatment, acute renal insufficiency, hemolysis, post blood transfusion recovery, spinal marrow, ischemic brain damage, congestive cardiac diseases and neurological injuries. EPO has been shown to prevent apoptosis, balance inflammatory responses, fuel angiogenesis and stimulate the engagement of stem cells [5-8, 11].

As an endogenous cytokine, EPO possesses a specific structure that allows it ability to function (Figure 1). The gene encoding EPO is located in chromosome 7q11-22. Its encoding genomic deoxyribonucleic acid (DNA) consists of four introns and five exons. Transcription occurs resulting in a mature EPO transcript. Following, this transcript is translated into a polypeptide chain. This polypeptide chain then undergoes post-translational modifications to give a final EPO structure. Before these post-translational modifications, the original EPO peptide structure is estimated to consist of 193 amino acids and posses a mass around 18 kilodalton (kDa). The post-translational alterations modify the structure of EPO through glycosylation, the formation of disulfide bonds and a freeing of its peptide chain. More specifically, glycosylation includes the attachment of N-linked oligosaccharides to Asn-24, Asn-38, and Asn-83. Glycosylation also fixes Ser-126 with an acidic O¬-linked oligosaccharide. Post-glycosylation, these new additions constitute 40% of EPO's final molecular weight. The glycocomponent of EPO works to maintain the protein's stability, biosynthesis, secretion and solubility. These are important characteristics and therefore, put importance on EPO's structure derived in part from glycosylation. Furthermore, another stabilizing factor is the disulfide bonds that are formed within EPO. One assembles between Cys-7 and Cys-161 while another develops between Cys-29 and Cys-33. Simultaneous to the formation of these two disulfide bridges, a 27 amino acid signal peptide of the N-terminal hydrophobic secretory sequence is removed. This results in the change of 193 original amino acids to 166 amino acids after post-translational modifications. Relating to the C-terminal, Arg-166 is assumed to be freed before the EPO protein is discharged into circulation. After these processes, the final structure of EPO results, containing a mass of approximately 30 kDa, bearing the 166 amino acids previously mentioned. These internal, necessary processes yield EPO ready for function [1- 3, 6, 13- 15].

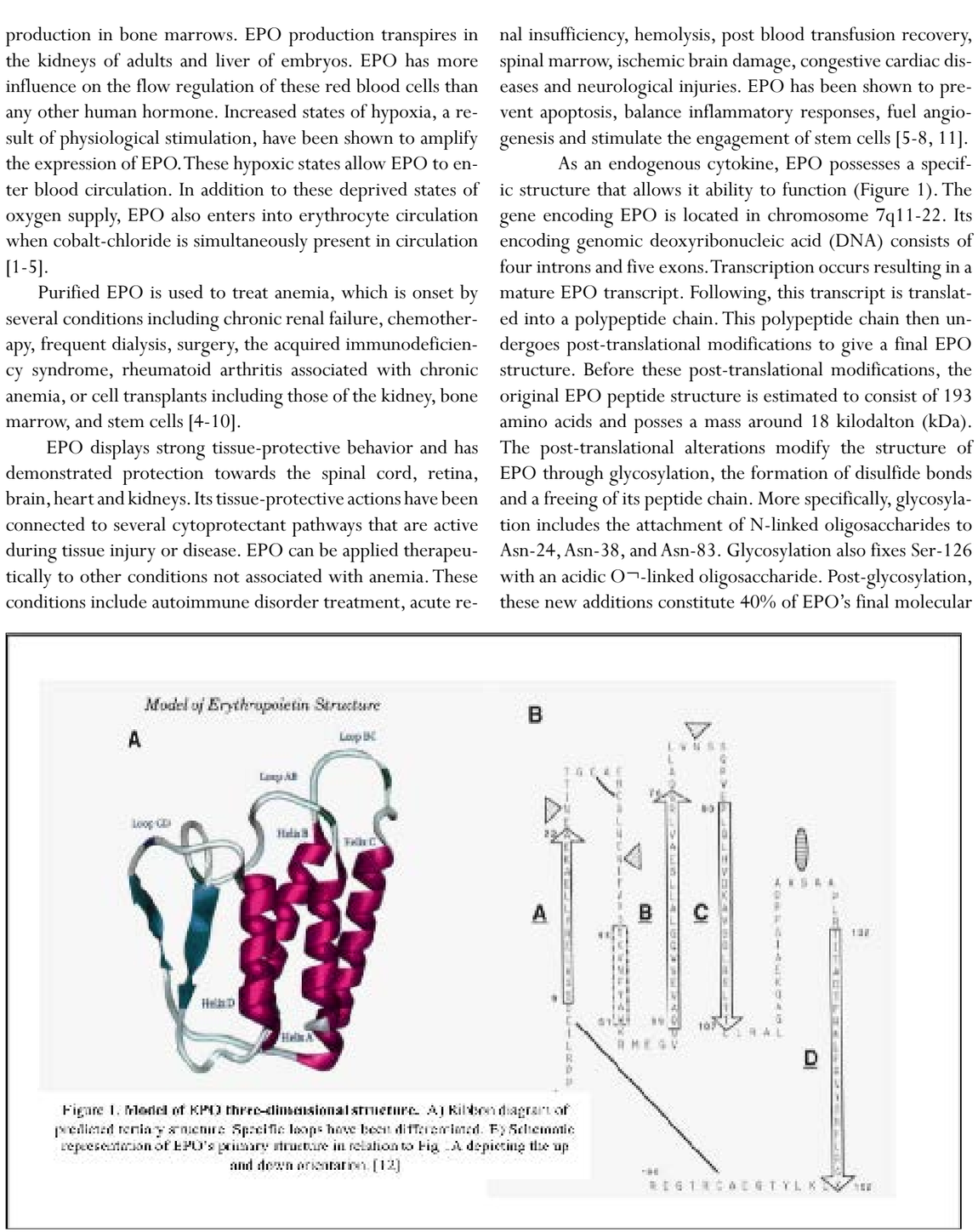


**Figure 1. Model of EPO three-dimensional structure.** A) Ribbon diagram of predicted tertiary structure. Specific loops have been differentiated. B) Schematic representation of EPO's primary structure in relation to Fig. 1A depicting the up and down orientation. [12]

In 1984, the first successful cloning of EPO was executed by Lee-Huang and coworkers [16]. This performance allowed victorious transformation of the cloned EPO in mammalian cells [3, 17-18]. Since then, EPO has been produced in yeasts [19], insect cells [20], bacteria [16] and in the milk of transgenic pigs [21] and goats [22] with varying results on efficiency of product generated. Another way that EPO has been produced is through transgenic plants [5, 23-27], although with limitations, as explained later. Transgenic plants provide a much cheaper process to produce recombinant EPO (rhEPO), which is eminent because of the high marketability of this leading biopharmaceutical glycoptotein. In 2003, its volume of business encompassed 8 billion US$, making rhEPO the foremost biopharmaceutical on the market. Therefore, the desire for efficient, rapid and economical production of this medical protein is an aspiration in the scientific community [10, 22, 25].

## *Transgenic Plant-Based Systems*

To improve traits of substances and the vitality of crops, continued development of genetic engineering technologies is necessary. New transformational strategies impact fundamental research along with agricultural biotechnology. This technology can allow for the precise and feasible growth of desired traits. Transgenic plant-based systems are included in these genetic engineering technologies and are defined as the transfer of appointed DNA to plant cells and consecutively, the regeneration of full plants. Therefore, it is important for transgenes to be capable of self-assimilation into the genome of the plant being used for transgenesis. The ability of these cells to regenerate into a whole plant while bearing these new characteristics is equally important [23-24].

The genetically engineered use of plants for transgenesis depends on differing factors. These factors include exonuclease activity, interaction of host factors, chromatin accessibility, DNA replication and repair activities. Transgenic plants have been utilized to produce agricultural, industrial and pharmaceutical proteins. Although many other means of producing recombinant proteins have been utilized, such as mammalian cultures, transgenic animals and microorganisms, production of these recombinant proteins is not always desired due to their immense cost of production and risks of contamination with human pathogens. Instead, plant-based systems offer many advantages for production when compared to mammalian and other systems for transgenic growth, including cost efficiency. This advantage alone may outweigh the authenticity of non-plant-based production. Additionally, the use of mammalian cultures is limited in production aptitude due to necessary complex reactors, maintains doubts as to the safety associated with its processes, exhibits possible harmful effects on host organisms and has questionable ethical acceptability among humans. In contrast, using transgenic plant processes to grow recombinant proteins is desirable because the use of these plant production systems offers safe production methods with rapid scalability, ability for large-scale production, ability to attend to synthesis of intricate proteins and they have a voidance of human pathogen interference that is linked to recombinant growth of proteins within mammalian cell cultures. These pathogens include impurities such as animal viruses, prions, toxins, mycoplasmas and other potentially hazardous substances. Those benefits are all in addition to the inexpensive means of production of these plant-based systems [24, 28-37].

Plant-based systems also posses a capacity for biomass production that surpasses any other type of production system. Their generation can yield several metric tons per hectare (ha), depending on the plant utilized for production. Plant expression systems have been used for production of pharmaceutically important serum proteins, antibodies, cytokines, potential vaccine antigens and lysosomal enzymes along with other proteins [37-39].

Nevertheless, production of rhEPO in plants is still not commercially feasible and mammalian cell systems are still employed to produce this and most valid human therapeutic proteins.

Although differences exist relating to variables involved, such

as plasmid vectors and the promoter sites employed, many experiments have been performed with the objective of producing rhEPO in differing crops through transgenic plant-expression systems.

The laboratory of Pasquali and coworkers [23] utilized tobacco and rice crop for the production of rhEPO. The employment of rice crops proved incapable for plant transformation, and consequently incapability of rhEPO production. It is thought that failure resulted from toxic effects due to the strong expression of the rhEPO. Employment of the tobacco crop for rhEPO production proved possible plant transformation, and recombinantly produced proteins exhibiting normal morphology and competence of rhEPO production. However, even though the results proved achievable in tobacco, the findings showed very low concentrations of produced rhEPO. The low efficiency was determined because only two out of 100 tobacco leaf discs resulted in successfully generated transgenic lines of tobacco containing rhEPO. Characteristics of EPO were exhibited; however, the low quantity of growth does not exhibit commercially feasible production of rhEPO within tobacco. Additionally, the inconsistency associated with tobacco use was further exhibited when attempts employing 200 tobacco leaf discs were performed and regeneration of rhEPO was not at all possible.

Cheon and coworkers [26] have similarly transformed and regenerated plant crops with EPO. They employed Arabidopsis thaliana and tobacco. However, T0 plants exhibited male sterility and malformations. Overexpression of EPO was shown here causing vegetative growth retardation, irregular arrangement of leaves in rosettes, bloom slowing, sterility and distorted flower buds. Therefore, even though transformation and reproduction were successful, commercial feasibility does not exist due to plant irregularities that would constrain ease of production and distribution.

Matsumoto and coworkers [27] successfully transferred and developed EPO within the plant genome of tobacco crop. However, the productivity of the cultured tobacco cells was too low for analysis of its biological functions. Their results indicated that their rhEPO is unstable due to deglycosylation occurring from processing, and therefore resulted in low productivity. The feasibility of transgenic plant production of rhEPO, therefore, proved impractical for commercial distribution as the other examples discussed.

## *Canola*

Canola, more scientifically known as Brassica napus L. (Figure 2), is a temperate oilseed crop that is vital worldwide as a source of plant oil and developmental products rich in protein. Canola is derived from the early, standard rapeseed crop. Through the hybridization of Brassica rapa and Brassica oleracea, the allotetraploid of B. napus was formed. It is compatible with more than 15 differing mustard species. In oil production, soybean is globally the largest producing crop. Following soybean is canola, making it the second largest crop for oil production worldwide. It is estimated that canola inhabits 5.9 Mha internationally [41-42].

A B

Figure 2. **Canola** A) *B. napus* seeds B) *B. napus* flower [40]

In relation to the genetic engineering technologies of plants, canola was amidst the initial crops to be genetically modified. Agrobacterium tumefaciens is frequently employed to transform

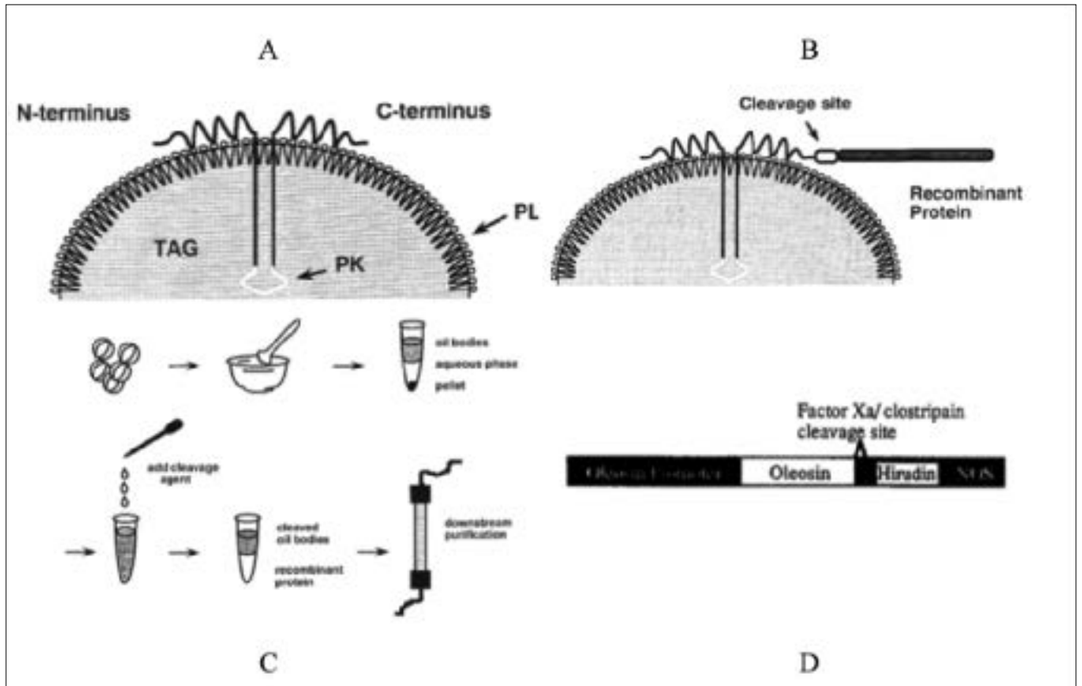


Figure 3. **Oleosin-Fusion Technology** A) Schematic diagram of proposed configuration of an oleosin protein embedded in an oil body. Triacylglycerol (TAG), Proline Knot (PK), Phospholipid Layer (PL). B) Representation of oleosin-protein fusion. C) Oleosin partitioning strategy of recombinant proteins. [37]. D) Expression cassette constructed by Moloney *et. al.* [87] for his experiments for the production of recombinant oleosin-hirudin proteins. Cassette includes oleosin promoter, oleosin encoding sequence, peptidase cleavage site, hirudin coding sequence and *Tnos*.

canola; however, canola has been transformed with other methods such as polyethylene glycol-mediated (PEG-mediated) DNA uptake, electroporation, microprojectile bombardment, microinjection, protoplast transfection and microspore transfection. These genetic transformation methods have been successfully employed to introduced herbicide, insect, fungi resistance, oil and proteins into the genome of canola. The A. tumefaciens-mediated transformation is the preferred method of transformation with canola because it encompasses an ease of execution and possesses great cost efficacy [43-73].

The competence of A. tumefaciens-mediated transformation depends on plant cultivar type, plant explant age and the components existing in plant and bacterial culture media. Improvement of current cultivars, formation of new cultivars, and bettering environmental circumstances for transformation are always desired. Recently, utilizing specific genetically modified methods, experiments have been performed with the intent of making canola tolerant to heavy metals and toxic compounds. Success would improve the suitableness of canola for use in phytoremediation. Equivalently ambitious and recent research aims the employment of canola to improve the production of biofuel and usability for the production of pharmaceutically active proteins and edible vaccines [41, 43-44, 74-79].

## *Oleosin-Fusion Technology*

Oilbodies are spherical chambers present in oil-producing plant cells for the storage of triacylglycerols (TAGs), phospholipids and proteins. These compartments are also referred to as spherosomes, but the term oilbodies is more commonly used. TAGs are lipids gathered by oilseeds for the purpose of supplying energy to their seedlings after germination. They are gathered within oilbodies during the growth of pollen and seeds. Oilbodies are cell organelles that develop within

the endoplasmic reticulum (ER). ER is also responsible for the production of TAGs. Depending on the type of oilseed in question, a seed's oilbodies may be found in different spatial locations. These organelles of albuminous oilseeds are located in the endosperm. This differs from the oilbodies of exalbuminous oilseeds, which can be found in its embryonic axis and cotyledons. Still different, the oilbodies of monocotyledonous species, including cereals, are found in the scutellum. TAGs are also stored in structures similar to oilbodies in the tapetum, pollen grains and oleaginous fruits [80].

Oilbodies have been shown to vary in diameter depending on the plant species where they occur. Their diameter normally ranges from 0.5 to 2 mm (micrometers). This range is applicable to plants whose oilbody lipids are predestined for use as energy following a dehydration process. When the lipids of a plant's oilbodies are not predestined for use as energy, their oilbody diameter can reach 20 mm. This being said, the size of oilbodies directly relates to their function [81, 82].

Oilbodies function in close proximity to one another when a seed is in its concluding stages of seed maturation. During these final steps, water potential decreases, and oilbodies are therefore compressed into one another as they encounter cytoplasmic compression. During this encounter, the organelles oppose coalescence and consequently maintain their small separate unit compositions [83].

In plants where oilbody lipids are predetermined for energy use, it is assumed that the oilbodies maintain their individual composition in order to provide a high surface-to-volume ratio. This would in turn provide access by lipases during germination and access to the energy needed. The mesocarps of oleaginous fruits are examples of tissues that do not go through this dehydration process for energy supply and therefore, their oilbodies may exhibit a larger diameter [81, 82].

The structure of the oilbody has been established through various scientific analytical processes. It has been determined that a phospholipid monolayer encompasses these organelles. In turn, its aliphatic chains are aligned to the triglyceride lumen of the plant while its phosphate groups are directed towards the cytoplasm. Chemical and ultrastructural analysis has uncovered this orientation. The weight of protein located within seed oilbodies has also been established through chemical analysis, being equivalent to 1-4%. The amount of total seed protein is also dependent on the plant species. For example, the oilbodies of peanuts contain relatively 0.3%, while those of canola consist of approximately 20% [80, 84-86].

The major proteins located within oilbodies are oleosins. They are unique to oilbodies according to subcellular fractionation experiments and immunocytochemistry. They are found throughout oilbodies and normally exist as two or more isoforms, categorized as either high or low molecular weight forms. The structure of oleosin is what makes it so unique. It is divided into three structural domains. The first is an N-terminal amphipathic domain. The second is a central hydrophobic core. The third is a C-terminal amphipathic domain. The central domain consists of a long hydrophobic core that consists of a proline knot. This proline knot is a distinctive 12-amino acid motif. This unique structure of the central domain is vital for the accurate targeting to oilbodies. The other two structural domains mentioned border this central hydrophobic core. These oleosins are secured to the oilbodies that encompass them through their central hydrophobic domain. The hydrophilic N- and C-terminal borders are therefore exposed to the cytoplasm (Figure 3A). This movement has been discovered by protease protection assays. Even though not all is known about oleosins, through experimentation, it has been shown that the stability of oilbodies is dependent and based on its amino acid sequence. Additionally, oleosins may play a part in lipase attachment [84-86, 88-95].

Therefore, the basic correlation of oilseeds, oilbodies and oleosin can be summed up to say that due to amphiphilic structure, oleosin proteins are rooted to oilbodies, and these oilbodies are found within oilseeds. The oilbodies within oilseeds are simple organelles. The oilbodies posses TAGs and are encompassed in a phospholipid monolayer. Oleosins are distinctive proteins fastened into and surfacing this monolayer [96].

The characteristics of oilseeds, oilbodies and oleosin proteins allow for the existence of oleosin-fusion technology. Oleosin-fusion technology is a modified transgenic plant-expression method that employs oilseeds, oilbodies and oleosin proteins for recombinant protein production. This method varies from standard transgenic-plant systems because with this technology the coding sequence of the protein desired for transgenic production is positioned in a plasmid adjacent to a peptidase cleavage site that is proximal to the oleosin coding sequence. This configuration is framed by a promoter and terminator sequence. After transformation and reproduction has occurred, a protein consisting of rhOleosin (recombinant oleosin) and rhPOI (recombinant protein of interest), separated by a peptidase cleavage site, will be produced (Figure 3B). A flotation centrifugation process (Figure 3C) occurs that separates the oilseeds, which contain the rhOleosin-rhPOI produced, into three distinct fractions. The oilseeds are crushed pre-centrifugation and centrifuged with an aqueous buffer. Post-centrifugation the fractions obtained consist of a pellet of insoluble material, an aqueous phase containing soluble cell components and an upper layer comprised of rhOleosin and the associated rhPOI. Resuspension of the oilbody in a fresh buffer will follow, allowing for repeat of the centrifugation process to further purify this layer. Following, through the peptidase site, enzyme usage cleaves the rhOleosin-rhPOI protein. A concluding centrifugation will allow recovery of the rhPOI devoid of the rhOleosin. Moloney et. al. [37] has shown that this process has a success rate greater than 90% in the isolation of rhPOI.

In addition, Boothe et. al. [37] and Moloney et. al. [87] have used oleosin-fusion technology to produce the protein hirudin (Figure 3D), a medicinally used anticoagulant peptide that is naturally produced in the leech Hirudo medicinalis. Canola was their crop of interest. Results from experimentation showed that rhPOI through this technology can be specifically localized to the oilbody fractions, display short and long-term stability, express their high-value protein peptides and possess correct protein folding structure, consequently exhibiting positive rhPOI activity.

**Timely Objectives**

| *Year 1* | *Year 2* | *Year 3* |
|---|---|---|
| Adaptation of human EPO coding sequence | Introduce pFINAL into *A. tumefaciens* | Oleosin-EPO cleavage |
| Cloning of *A. thaliana* oleosin gene | Generation of transgenic canola | Isolation of active rhEPO |
| Formation of pFINAL | Oleosion-EPO production in canola | |

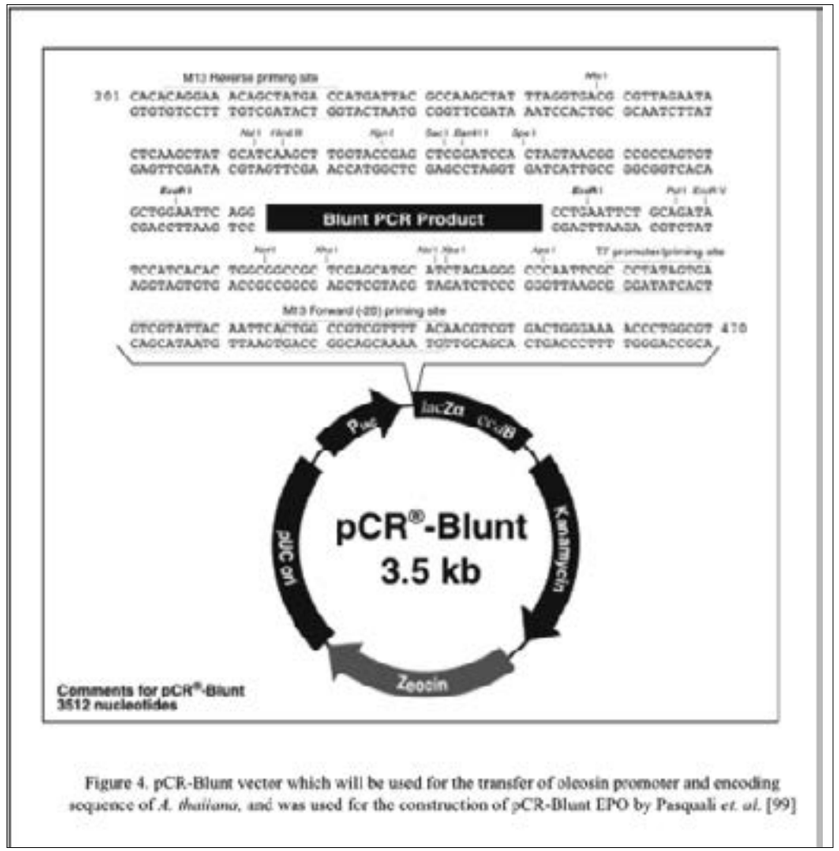


Figure 4. pCR-Blunt vector which will be used for the transfer of oleosin promoter and encoding sequence of *A. thaliana*, and was used for the construction of pCR-Blunt EPO by Pasquali *et. al.* [99]

## Justification and Objectives

### *Justification*

The high cost of using mammalian cell systems compared to the low cost of utilizing plant-expression systems for the production of recombinant proteins creates the desire for continued discovery of successful transgenic plant-expression systems. Therefore, due to the wide variety of capabilities EPO possesses, a plant-expression system to produce rhEPO that will not exhibit flaws to its capabilities or the plants utilized for transformation is desired within the medical community. Oleosin-fusion technology has proven to be successful in transgenic growth of recombinant protein

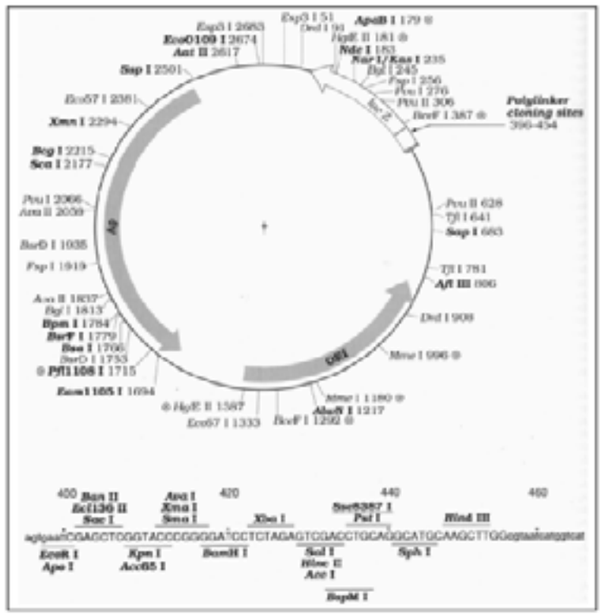


Figure 5. pUC19 vector which will be used for the construction of the expression cassette of interest, which will include the oleosin promoter sequence, oleosin encoding sequence, peptidase cleavage site, EPO coding sequence and *Tnos*. [100]

that exhibits normal morphology and capabilities of the plant and recombinant protein in question. Therefore, the common toxicity associated with plant-expression systems for production of rhEPO may be expelled with the use of oleosin-fusion technology.

The performed experiments of Moloney et. al. [87] have displayed unprecedented efficiency in expressing the protein hirudin in canola through oleosin-fusion technology. His experiment relates directly to the oil crop of interest, canola (B. napus). Here, the reproduction of hirudin-oleosin in canola is proposed, making modifications to suit the biopharmaceutical protein of interest, EPO. It is proposed to only make the minimal modifications necessary to see if EPO can be transgenically synthesized using oleosin-fusion technology in canola following the processes of Moloney et al. [87] as a guide.

## *Objectives*

### General Objective

Successful production of rhEPO by methods employing oleosin-fusion technology within transgenic canola.

### Specific Objectives

- Adapt the human EPO encoding sequence in order to be inserted in an expression cassette for plant transcription in fusion with the A. thaliana oleosin gene sequence.
- Cloning of the A. thaliana oleosin gene, including its promoter sequence.
- Transformation and inclusion of adequate restriction sites to construct a final binary vector (pFINAL) containing the expression cassette of an oleosin promoter, oleosin coding sequence, peptidase site, EPO coding sequence and nopaline synthase terminator (Tnos).
- Introduction of pFINAL into A. tumefaciens through electroporation.
- Successful regeneration of transgenic canola plants through transformation of petioles with A. tumefaciens containing pFINAL.
- Production of the oleosin-EPO fusion protein within canola.
- Cleavage of the oleosin-EPO fusion protein that was produced within canola.
- Successful activity of isolated rhEPO after cleavage from its oleosin protein attachment.

## Technical Strategies

Embodying Moloney et. al. [87] as a template, here is proposed the means to perform this experiment.

The plasmids specifically needed for this experiment are pCR-Blunt EPO [Invitrogen], pCR-Blunt [Invitrogen], pUC19 [New England BioLabs], pBI121 [87], pBluescript KS+ [87] and pCGN1559 [87]. Plasmid pCR-Blunt EPO and pCR-Blunt are presently available in LBMV and therefore, do not need to be purchased. The construction of pCR-Blunt EPO was executing using an oligonucleotide overlapping technique with the full coding sequence of human EPO and based on available mRNA sequence at GenBank [23, 97]. Purchased pUC19 will be isolated from Escherichia coli, strain ER2272, through standard plasmid purification procedures. Propositioned pBI121 originated from Clontech while Moloney et. al. did not address his origin of pBluescript KS+ and pCGN1559.

- To begin this experiment, pCR-Blunt EPO will need to be modified by the inclusion of SalI and PvuI restriction sites through polymerase chain reaction (PCR). Appropriate primers will be synthesized in order to introduce the modifications, using the whole plasmid as DNA-template.

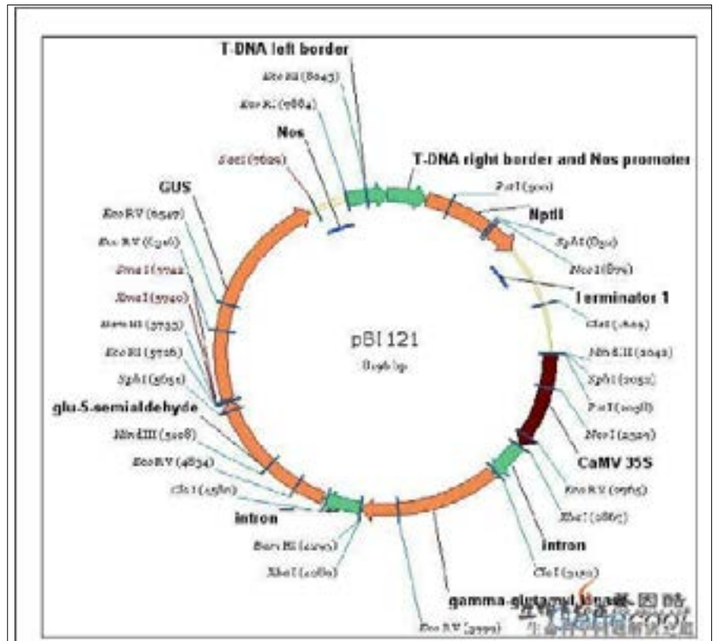


Figure 6. pBI121 vector which includes *Tnos* which will be cleaved with SacI and EcoRI enzymes for construction of the expression cassette. [101]

- Separately, total RNA from A. thaliana young siliques and seeds will be extracted. A. thaliana will be used because its gene bears 91% homology to B. napus oleosin and has shown to be successful in the oleosin-fusion experimentation when canola crop is employed for location of transgenic production [37, 87]. Extracted RNA will undergo cDNA synthesis. Primers flanking the oleosin promoter and coding sequence will be designed in order to amplify the gene through PCR. The oleosin promoter and coding sequences needed to be amplified will use the same primers employed by Moloney et al. [98]. Reverse transcription followed by PCR using the primers should yield enough copies of the oleosin promoter and coding sequence in order to be cloned into pCR-Blunt plasmid.
- Using an aliquot of the PCR product, agarose gel electrophoresis (AGE) should be performed to determine if PCR usage was successful. This process should be employed to determine success after every amplification by PCR.
- The amplified fragments of the A. thaliana genome will be ligated into pCR-Blunt (Figure 4). After cloning, the pCR-Blunt vector will contain the oleosin promoter and coding sequences.
- The ligated pCR-Blunt should be transferred to E. coli cells, have colonies inoculated in liquid medium and undergo plasmid miniprep. Following, endonuclease restriction digestion and AGE should be performed to determine if the transformation of the oleosin promoter and coding sequence into the pCR-Blunt vector was successful. This process employing transformation into E. coli cells, inoculation of colonies in liquid medium and plasmid miniprep followed by endonuclease restriction digestion and AGE for the determination of successful transformation from one plasmid to another should be performed after every fragmentation transfer.
- Restriction sites PstI, SalI, and PvuI need to be added to the transformed pCR-Blunt through PCR as Moloney et al. [98], changing the primer sequence.
- The fragment of interest, which includes the oleosin promoter and oleosin coding sequence, needs to be cloned into the SmaI site of the pUC19 plasmid (Figure 5). After cloning, the pUC19 vector will contain the oleosin promoter and coding sequences. The resulting plasmid will be called pOBIL.
- Following, the Tnos terminator sequence of pBI121 (Figure 6) will need to also be transferred into pOBIL. The Tnos will be cleaved using SacI and EcoRI enzymes. After AGE, the Tnos fragment will be purified from gels by the freeze-squeeze method of elution.
- The Tnos will be cloned through digestion with SacI and EcoRI enzymes and ligated into the pOBIL. Plasmid pOBIL with the Tnos will be called pTERM.
- The EPO fragment will be excised from pCR-Blunt EPO with SalI and PvuI enzymes and ligated into these restriction sites of pTERM. Therefore, the translation of the resulting mRNA in plant cells will start at the start codon of oleosin and it will finish at the stop codon of EPO. This modified pTERM vector will now also have the addition if an EPO coding sequence. This plasmid will be called pOBHIRT.
- A sequence encoding a Factor Xa/clostripain cleavage site should be interposed to facilitate the purification of rhEPO. It should be inserted between the oleosin and EPO coding sequences following above formation of pOBHIRT. This step is important because when protein is synthesized within canola, the protein will consist of the rhOleosin protein adjacent the peptidase cleavage site, which is subsequently alongside the

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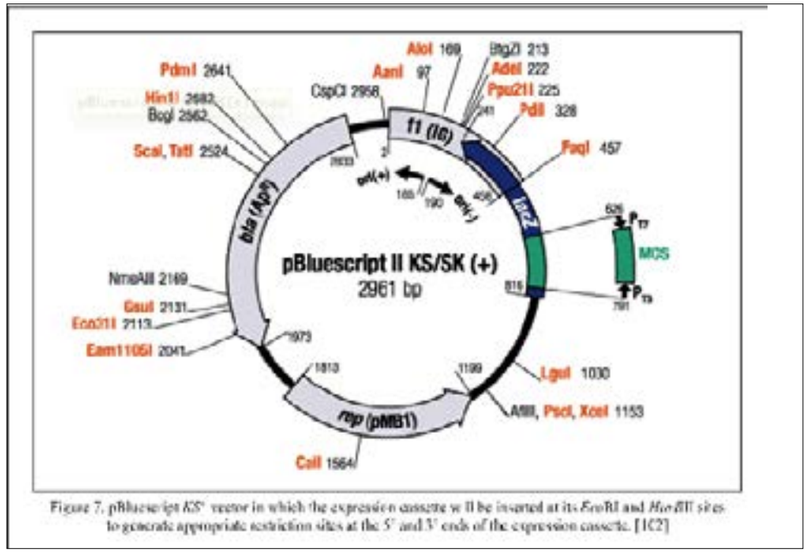
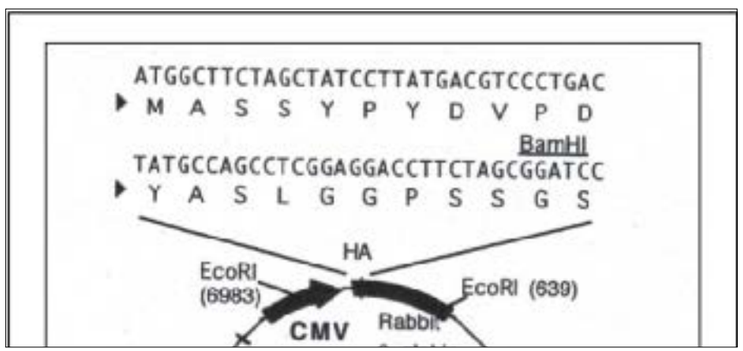



Figure 7. pBluescript KS+ vector in which the expression cassette will be inserted at its *EcoRI* and *HindIII* sites to generate appropriate restriction sites at the 5' and 3' ends of the expression cassette. [102]

rhEPO protein. This peptidase site will allow the cleavage of rhOleosin from rhEPO, which is a crucial step in the methods of oleosin-fusion technology.

- The vector pBluescript KS+ (Figure 7) shall be employed to generate appropriate restriction sites at the 5' and 3' ends of the fragment of interest [87]. This expression cassette fragment (oleosin promoter, EPO coding sequence, peptidase site, oleosin coding sequence and Tnos) within pOBHIRT will




be transferred to pBluescript KS+ through excision and further ligation with EcoRI and HindIII. The resulting vector will be called pBLUE.

- The binary vector pCGN1559 (Figure 8) shall be employed due to its ability to replicate within A. tumefaciens. The expression cassette fragment now in the pBLUE vector will be transferred into pCGN1559 at its PstI site. The resulting construct plasmid is designed to encompass the necessary sequence for canola transformation via A. tumefaciens transformation. This vector will be called pFINAL.

- The next step is the introduction of pFINAL into A. tumefaciens. This introduction shall be performed using electroporation according to Dower et. al. [104].

- A. tumefaciens harboring pFINAL will be used to transform canola. This shall be performed according to Moloney et. al. [105], employing plant's petioles. The tissues will grow and be maintained in a culture room. The B. napus seeds will be sterilized with commercial bleach with shaking. Seeds will be washed with sterile double-distilled water. They will be placed on Murashige-Skoog medium and then in a growth room. A. tumefaciens will also be grown in Murashige-Skoog medium. Cotyledonary petioles will be used for transformation. After growth, their cut ends will be dipped into the A. tumefaciens suspension. The tissues will then be transferred into Murashige-Skoog medium. The regenerate shoots will be obtained after a few weeks. They will be cut and placed in Magenta jars onto Murashige-Skoog medium. Once roots emerge, plantlets will be transferred to potting mix. Following, they will be placed into a misting chamber. Leaf samples will be taken for neomycin phosphotransferase assays. A few weeks later, the plants will be transferred to a greenhouse where they will flower, self-fertilize and set seed.

- Canola can be checked for transformation by checking for the presence or absence of Hygromycin B resistance. Resistance to this antibiotic will be checked because the Hygromycin B resistance gene is present between the left and right bounds of the pCGN1559 vector. Where Hygromycin B resistance is shown, the pFINAL has successfully been introduced. Where Hygromycin B resistance is not shown, pFINAL was not successfully introduced.

- Total DNA and RNA will be extracted from transgenic and non-transgenic (control) canola plants through kits purchased for nucleic acid extraction [Agilent Technologies] and performed according to Verwoerd et. al. [106]. Respectively PCR and RT-PCR employing EPO-specific primers will be performed in order to prove the transgenic state of plants and the expression of the transgene at the mRNA level as performed by Moloney et. al. [87].

- Western blot analysis will be used to determine the presence of the oleosin-EPO fusion protein in canola seed protein extracts. Total proteins will be extracted from seeds according to Moloney et. al. [87]. Antibodies able to recognize EPO and/or oleosin will be acquired from Moloney and coworkers or Jo Ross at John Innes Centre, UK (who generously donated the antibodies to Moloney et. al.) or produced in rabbits according to Ross et. al. [107].

- Following this, the oleosin-EPO fusion proteins shall be cleaved and rhEPO will be checked for activity. Separation will be employed through flotation centrifugation and three separate divisions of the total seed protein will result – a water-soluble division, a sedimenting division and an oilbody division. The cleavage should be performed through the Factor Xa/clostripain cleavage site. Further processing, employing crushing, centrifugation and resuspension as described in the bibliographic review section will result in the isolation of rhEPO.

- The rhEPO's biological activity will be analyzed by its ability to prevent the cellular death of renal tubular epithelial cells (TECs). This will be performed according to methods of Du et. al. [108]. The method is based on the demonstration by Matsumoto et. al. [109] and Weise et al. [110] that rhEPO possesses tissue-protective bioactivities. This was demonstrated by its capability to lower the susceptibility of renal TECs to cytokine-induced cell death. Therefore, employing renal parenchymal cells, which are highly composed of TECs, would be useful for observing the capacity of renal TECs to resist cell death. The ability of rhEPO to prevent cellular death of renal TECs will indicate that rhEPO is produced and purified in an active form.

## Laboratory Infrastructure

This experiment shall be performed in the Laboratório de Biologia Molecular Vegetal (Plant Molecular Biology Laboratory – LBMV) of the Center for Biotechnology (CBiot) of the Universidade Federal do Rio Grande do Sul (UFRGS), under the responsibility of Dr. Giancarlo Pasquali. The accessibility of other facilities of common use, located within CBiot, shall also be employed. The LBMV posses much of the equipment necessary for the execution of this proposal, and the personnel has experience with performing transgenic-plant expression processes and molecular biology protocols.

To perform this experiment, financial funding for a three-year time period of work is necessary. Based on an R$ 25,000.00 annual budget, a total inquiry of R$ 75,000.00 will be adequate to carry out this research proposal. The requested financial assistance shall support airline tickets, maintenance and repair of LBMV's equipments, purchase of needed plasmids, reagents, media, glassware, plastic ware and other substances specific to this proposal of research, along with basic materials and equipment required for experimentation. These basic necessities include an automated DNA sequencer, mono and multichannel micropipettes, photodocumentation systems, refrigerators, ultra-freezers, freezers, sources for electrophoresis with high and low voltage, variety of tanks and accessories for electrophoresis, semi-analytical and analytical balances, thermocycler microtube and microphate for conventional PCR, horizontal and vertical laminar flow hoods, water baths, microcentrifuges, microplate rotor, water purification system, ice machine, vertical autoclave, drying ovens and vortex shaker vibration.

## Expected Results and Discussion

### *Expected Results*

Four main products of scientific and commercial value are expected from the successful accomplishment of the present work. First, a binary plasmid vector containing an expression cassette made up of an oleosin promoter, oleosin coding sequence, peptidase site, EPO coding sequence, and Tnos shall be constructed. This plasmid will be named pFINAL and will be protected by intellectual property. Secondly, A. tumefaciens cells harboring binary vector pFINAL shall occur. Third, transgenic canola plants and seeds will be produced, containing measurable values of rhEPO in active form. Finally, rhEPO in active form is expected, that can be chemically defined and tested in cells and animal models of anemia as well as first clinical trials. The active rhEPO will prevent the cellular death of renal tubular epithelial cells (TECs).

### *Discussion*

This proposal is important to the scientific community because oleosin-fusion technology is a new variation to the traditional means of employing plant-expression systems. Experimentation with this method will present more data, in return increasing understanding of this technique. Additionally, oleosin-fusion technology has been shown to produce very stable recombinant proteins according to Boothe et. al. [37]

and Moloney et. al. [86, 87, 96, 111] on several reported and documented occasions from 1995-2006. If supported by this proposal, the longevity of this transgenic-plant production process will be more credible and add to the alluring quality to its usage. In addition, as mentioned earlier, EPO is a highly desired and beneficial biopharmaceutical protein. If this proposal is successful, the cost-efficient means of producing rhEPO by this method will be promoted, adding commerciality to the benefits of this technology [87].

Moloney et. al. [112] have produced patents pertaining to the use of oleosin-fusion technology to commercially produce recombinant proteins. Two of these patents are referenced as #7,786,352 ("Methods for the production of apolipoproteins in transgenic plants") and #7,666,628 ("Preparation of the hererologous proteins on oil bodies"). However, EPO was not produced or proposed by the employment of the technique. Therefore, and due to the attractiveness and capability of rhEPO within the medical community, the benefits of rhEPO's successful production through implementation of this technique possesses benefits and intellectual novelty that may result in gains to all researchers and institutions involved.

In conclusion, whether the desired outcomes of this proposal will be produced or fail to be produced, there is no doubt that this proposed experiment will greatly contribute to the insight of its practice, therefore furthering knowledge of the scientific community in relation to recombinant protein production with the oleosin-fusion technology.

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## Abbreviations

AGE – agarose gel electrophoresis
Arg – arginine
Asn – aspargine
CBiot – Centro de Biotecnologia/Biotechnology Center
cDNA – complementary deoxyribonucleic acid
Cys – cysteine
DNA – deoxyribonucleic acid
EPO – erythropoietin
ER – endoplasmic reticulum
ha – hectare
kDa – kilodalton
LBMV – Laboratório de Biologia Molecular Vegetal/Plant Molecular Biology Laboratory
Mha – million hectares
mm – micrometer
mRNA – messenger ribonucleic acid
Tnos – nopaline synthase terminator
PCR – polymerase chain reaction
PEG – polyethylene glycol
R$ – Brazilian reais
rhEPO – recombinant human erythropoietin
rhOleosin – recombinant oleosin
rhPOI – recombinant protein of interest
RNA – ribonucleic acid
Ser – serine
TAGs – triacylglycerols
TEC – tubular epithelial cells
UFRGS – Universidade Federal do Rio Grande do Sul
US$ – American dollars

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