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Review

Pharmaceutical proteins produced in plant bioreactor in recent years

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Plant bioreactor, also called molecular farming, has enormous potential to produce recombinant proteins infinitely. Products expressed in plants have natural physico-chemical properties and bioactivities. Plant bioreactor could be a safe, economic and convenient production system, and can been widely applied in industries and agricultures, especially in the life science and pharmaceutical industry. The application of transgenic plant in the production of vaccines, antibodies and pharmaceutical proteins has become a hot point in the plant genetic engineering in recent years. However, there are some limiting factors of application such as yield, downstream processing and so on. Here we review and discuss the advantages and research progress for the pharmaceutical plant bioreactors in recent three years, focusing on the existing problems and new strategies in this area.

Key words: Plant bioreactor, pharmaceutical proteins.

INTRODUCTION

Plant genetic transformation is being perfected because of the rapid development of molecular biology. Faced with increasing demand of protein diagnostics and therapeutics, the application of transgenic plants in the production of pharmaceutical proteins has become a well-recognized and important field of biopharmaceutical science with promising economic potential. Plant bioreactor as a safe, convenient and economical production system for recombination protein has been widely accepted by the public. As showed from the recent two decades, a variety of valuable proteins can be effectively expressed in plants such as human serum protein, growth regulators, antibodies, vaccines, industrial enzymes, biological polyose and molecular biological reaction reagents. A series of successful experiments make it possible to produce foreign proteins by genetically modified plants, which show tremendous market prospect and commercial value. This is known as molecular farming. The first generation genetically modified plant-derived products have entered comercialization stage. Plant bioreactor has many potential advantages for the production of recombinant proteins compared with microbial and animal cell culture systems:

1) Cost on an agricultural scale to produce the raw material is low and it is easy to scale up.
2) Genetic determinant of progeny from self so polygene can accumulate in plants (Liu and Wu, 2000).
3) The accumulation of recombinant proteins in seeds, tuber and fruits can be beneficial for storage obviating the influence of ambient temperatures. Especially the plant vaccine for oral deliver needs no exceptional storage conditions (Korban et al., 2002; Mason et al., 2002; Stoger et al., 2000).
4) The main disadvantage of bacterial expression is that post-translational modification processes do not take place. Plant-derived proteins are relatively safe because the absence of contamination by animal pathogens (Commandeur et al., 2003).
5) The proceeding approach for protein is relatively conservative. Plants have the ability to correctly carry out post-translational modification such as glycosylation, phosphorylation and amidation. Thus the immunogenicity and biologic activity of recombinant proteins are similar to those in higher animal cells (Daniell et al., 2001).

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PRODUCTION OF WHOLE ANTIBODIES AND ANTIBODY FRAGMENTS

Currently, production of full-size antibodies, Fab and F(ab')2 fragments (which contain only the sequences distal to the hinge region of a full-length antibody), single-chain Fv fragments (scFvs, which contain the variable regions of the heavy and light chains joined by a flexible peptide linker), chimeric antibodies such as IgG/A, single-domain antibodies and antibody fusion protein have gained remarkable achievement with plant bioreactor system.

Full-size antibodies

Hiatt et al. (1989) gave the first report describing the successful expression of heavy and light-chains and assembly of functional dimeric antibodies with biological activity in transgenic tobacco, which had become a breakthrough in the field of plant genetic technology. Generally, one of two different approaches has been employed to produce biologically active whole antibodies in plants: transformation of the heavy and light-chain genes separately into plants, followed by cross-pollination of the two transgenic parents to yield F1 individuals carrying both the heavy and light chain gene (Hiatt et al., 1989; Hein et al., 1991; Ma et al., 1994) and co-transformation of the heavy and light chain genes on a single expression cassette (During et al., 1990). By using the former strategy, recombination antibody can be produced at high yield which reaches 1 to 5% of total protein. The later strategy can be used to avoid offspring isolation. But selection of promoter and terminator should be cautious to ensure the co-expression of two different genes (Chen and Wu, 2005).

The feasibility of producing a protective, human-derived, monoclonal antibody directed against the protective antigen (PA) of *Bacillus anthracis* in plants was demonstrated by transient expression using agroinfiltration of *Nicotiana benthamiana* plants (Hull et al., 2005). As yet, IgG1, IgM, IgA, chimeric antibody IgG1/IgA and Fab genes have been successfully expressed in plants.

Antibody derivatives

At present, most reports focus on micromolecular engineered antibody which has simple molecular structure. It is of great superiority producing scFv compared with complete antibody and Fab which need rigorous fitting process (Borrebaeck, 1995). Nejad et al. (2005) produced transgenic tobacco plants that express an anti-*Salmonella enterica* single-chain variable fragment (scFv) antibody that binds to the lipopolysaccharide (LPS) of *S. enterica* Paratyphi B. The purified scFv was active as a dimer or higher-order multimer. The recombinant sdAb fragments with specificity for a cancer-associated mucin, MUC1 was successfully expressed in transgenic tobacco plants (Ismaili et al., 2007). Galeff et al. (2006) described the production of recombinant single chain fragments of antibody variable regions (ScFvs) to ErbB-2, which offered a useful reagent for *in vitro* biochemical and immunodiagnostic applications in oncology. Xu et al. (2007) cloned the gene encoding the variable regions of both the heavy (H) and light (L) chains of the murine monoclonal antibody (mAB) S19, and this gene (scFv1 9) was expressed in transgenic tobacco to produce a recombination anti-sperm antibody (RASA).

Low level of expression is the limiting factor for application of plant antibody. We can use appropriate regulatory elements in the expression construct, optimizing codon usage and enhancing the stability of the antibody to improve the expression level of recombinant antibody. Some complete antibody, Fab and ScFv are expressed in cells at different levels, which depend on the sizes of antibody frame and their own frame. Other considerations also exist. It is important to recognize that potential differences in activity and specificity might exist between native antibodies and recombinant antibodies produced in animal or human cells and plant-based systems. The difference in glycosylation pattern is the main reason which causes immunogenic potential. Thus, choosing different means of recombinant protein targeting is the key issue in production. Generally, full-size antibodies, large single-chain antibodies, and camelid heavy-chain antibodies, which contain an Fc region carrying glycosylation sites, should be targeted to the ER for glycosylation, disulfide bonding and proper assembly (Ko and Koprowski, 2005). Addition of KDEL retrieval signal will cause proteins accumulate at high level in ER through the secretory pathway with out any loss.

PRODUCTION OF VACCINES

The development of transgenic plant-derived vaccine depends on organic combination of plant gene engineering technology and organism immunologic mechanism. There are two approaches for production transgenic plant vaccine. One way is to separate and purify the foreign protein produced in plant tissue to a high standard for intravenous use. The other is to express the protein in edible part of plants without downstream processing for direct oral vaccine. As demonstrated from animal experiment, the plant antigen after isolation and purification still remains immunocompetence that gives rise to the production of specific antibody in animal. Plant oral vaccine can also induce mucosal and humoral immune response in intestine. Edible vaccine offers some distinct advantages over traditional vaccination, including low cost, convenient to store and ease to use, so that it becomes the research focus in genetically engineering

vaccine. Five groups of plant vaccines have been reported from previous studies: bacterial vaccine, virus vaccine, pregnancy vaccine, parasite vaccine and diabetes vaccine.

Shchelkunov et al. (2006) for the first time produced a kind of candidate oral vaccine against both HIV and HBV viruses which can elicit immune responses in test animals. Li et al. (2006) constructed a DNA containing the cholera toxin B subunit (CTB) gene genetically fused to a nucleotide sequence encoding three copies of tandemly repeated diabetes-associated autoantigen, the B chain of human insulin, and transferred it into low-nicotine tobaccos.

The fusion protein was successfully expressed and retained GM1–ganglioside receptor binding specificity, which provide an effective approach for preventing and treating autoimmune diabetes by inducing oral tolerance. Fitchen et al. (1995) expressed a 13 amino acid sequence of the murine zona pellucida ZP3 protein in modified tobacco mosaic virus (TMV) coat protein. Serum antibody recognizing ZP3 epitope was produced in mice through parenteral immunization with virus-like particles containing the hybrid coat protein purified from infected plant tissue. The plant vaccines expressed recent three years were listed in Table 1.

Although the expressions of recombinant vaccine in plants have achieved great progress, there are many problems to be solved before commercialization. First, host species amenable to plant production system are always model plants such as *Arabidopsis thaliana* and tobacco which may not be an ideal choice for edible vaccines to scale up. Producing vaccine in tobacco leaves will come with the associated risks of contamination with nicotine and other alkaloids. Second, oral vaccine may not survive the gastrointestinal conditions with certain loss of immunogenicity or degradation. Finally, low yield of foreign protein is a great drawback to overcome.

PRODUCTIN OF OTHER PHARMACEUTICAL PROTEINS

The transgenic plant is also used as bioreactor for production of cytokine, enzyme, biologically active peptide and other pharmaceutical proteins, which has been explored effectively during the past decade. Hirudin as the most effective thrombin inhibitor was successfully expressed in oil bodies of rape seeds, which was the first case of commercial production. As yet, there has been a wide range of valuable pharmaceutical proteins produced in plants. Examples include actrapid monotard, erythropoietin, interferon, lysozyme, human growth hor-mone, enkephalin, human epidermal growth factor, blood coagulation factor, interleukin, hemoglobin, serum protein, thymosin and so on. The pharmaceutical proteins expressed in plants recent three years were listed in Table 2.

PROBLEMS IN PLANT SYSTEMS

Low yield of foreign protein

Low level of expression of heterologous proteins in transgenic plants is a ubiquitous problem that most researchers have encountered. For plant vaccine, only by reaching a high expression level in host plant can the oral immune response be elicited. In order to achieve commercial quantity, the recombinant protein must reach 1% of total soluble protein when producing plant antiibody needing purification in plant system (Xiao et al., 2003).

Factors affecting the yield of recombinant proteins produced in a plant system include the potential of production host and expression system, level of transgene expression and stability of the recombinant protein. By combining above factors, some strategies are explored to enhance the expression level of foreign protein.

1) Protein targeting

Targeting the transgenic products to the subcellular compartment (cytoplasm, apoplast, endoplasmic reticulum, chloroplast, vacuole and others) may enhance the accumulation level in plants, which is confirmed by extensive research of independent expression system. Chloroplast transformation technology is a method to integrate the transgene into specific genome sites via homologous recombination, which may avoid gene silencing or position effects (Liu, 2007). Expression of foreign genes in chloroplast genomes can dramatically enhance the level of expression thanks to hundreds and thousands copies of genome. The highest protein yield reached 46.1% of total soluble protein by this transformation method (Daniell et al., 2005). Besides, the endoplasmic reticulum targeting will give a high yield of foreign protein that can increased by 10 to 100 times. In order to increase expression level of cholera toxin B subunit (CTB), Kim et al. (2006) optimized codon usage and fused the CTB gene with an ER retention signal, KDEL before transforming to lettuce plants. The results that CTB reached total soluble protein (TSP) levels of 0.24% in transgenic lettuce would provide a valuable tool for the development of edible vaccines.

2) Optimization of foreign gene

The usage frequency of genetic codon in plants is different from animals and microorganism. Using the preferred codon is an efficient way to improve accumulation level in plants. Moreover, optimization of mRNA stability and translational efficiency, and the removal of spurious AU-rich sequences that may act as cryptic splice sites are internal factors that affect the level of transgene expression (Schillberg et al., 2003).

Table 1. Recombinant vaccines expressed in transgenic plants (after year 2005).

Category	Antigen	Host plant	References
Virus vaccine	HBsAg	blnana	Kumar et al., 2005
	HBsAg	*Nicotiana tabacum* L.	Kumar et al., 2006
	HBsAg middle protein	*Solanum tuberosum* L.	Youm et al., 2006
	HBsAg	*Nicotiana tabacum* L.	Shekhawat et al., 2007
	HBsAg	*Lupinus luteus*	Pniewski et al., 2006
	infectious bursal disease virus	Rice	Wu et al., 2007
	Newcastle disease virus fusion protein rabies V virus nucleoprotein	*Zea mays* L. *Lycopersico-n esculentum*	Octavio et al., 2006 Perea et al., 2008
	Hepatitis C Virus (HCV)-Derived Epitope	*Nicotiana tabacum*	Piazzolla et al., 2005
	Tumor-associated colorectal cancer antigen Highly pathogenic avian influenza A (H5N1) virus hemagglutinin neuraminidase protein of Newcastle diease virus	*Beta vulgaris* Barley grain *Nicotiana tabacum*	Brodzik et al., 2008 Bruchmüller et al., 2007 Hahn et al., 2007
	Newcastle diease virus immune protein	*Solanum tuberosum*	Berinstein et al., 2005
	foot-and-mouth disease virus structural protein	*Nicotiana tabacum* L.	Li et al., 2006
	Foot-and-mouth disease virus structural polyprotein	Alfalfa	Santosa et al, .2005
	Foot-and-mouth disease virus structural polyprotein	*Lycopersico-n esculentum*	Pan et al., 2008
	Neutralizing epitope of porcine epidemic diarrhea virus	*Solanum tuberosum*	Kim et al., 2005
	Capsid proteins of rotavirus-like particles Rotavirus VP6 protein	*Lycopersico-n esculentum* Alfalfa	Saldana et al., 2006 Dong et al., 2005
	poliovirus peptide	*Nicotiana tabacum* L.	Fujiyama., 2006
	Norwalk virus capsid protein.	*Lycopersico-n esculentum*	Zhang et al., 2006
	Tuberculosis antigens	*Nicotiana Tabacum* L.	Dorokhov et al., 2007
	Severe acute respiratory syndrome (SARS)-CoV spike protein	*Nicotiana Tabacum* L.	LiHY et al., 2006
	Severe acute respiratory syndrome S protein	*Nicotiana Tabacum* L.	Pogrebnyak et al., 2005
	Avian reovirus sigma C protein	*Medicago sativa* L	Huang et al., 2006
	HIV capsid protein	*Lycium barbarum* L.	Du et al., 2005
	HIV(type 1)tat protein	*Lycopersico-n esculentum*	Ramirez et al., 2007
	HIV(type 1)tat protein	*Spinacea oleraceae*	Karasev et al., 2005
	Papillomavirus Type 16 Major Capsid Protein L1	*Arabidopsis thaliana*	Adab et al., 2007
	Human Papillomavirus Type 11 protein L1	*Nicotiana Tabacum* L	Kohl et al., 2007
	Measles virus mutants polyepitope	*Daucus carota* L	Bouche et al., 2005
Bacterial vaccine	Measles virus hemagglutinin protein cholera toxin B subunit	*Lactuca sativa* *Lactuca sativa* L.	Webster et al., 2006 Kim et al., 2006
	Actinobacillus pleuropneumoniae Toxin	*Nicotiana Tabacum* L	Lee KY et al., 2006
	Helicobacter pylori urease subunit B	*Nicotiana tabacum* L.	Gu et al., 2005
	Helicobacter pylori heat-shock protein A, epitopes of the diphtheria, pertussis and tetanus exotoxins	*Nicotiana tabacum* L. *Lycopersico-nesculentum*	Zhang et al, 2006 Soria-Guerra et al., 2007
	Lyme disease agent outer-surface protein A	*Nicotiana tabacum* L	Navarre et al, .2006
	Glycosylated F4 (K88) fimbrial adhesin FaeG	*Hordeum vulgare* L.	Joensuu et al., 2006
	Shiga toxin (type 2)	*Nicotiana tabacum* L	Wen.et al., 2006
	E. coli heat-labile enterotoxin B subunit	*Nicotiana tabacum* L	Kang et al., 2005
	Helicobacter pylori urease subunit B	*Oryza sativa* L.	Gu et al., 2006
Parasite vaccine	Cysticercosis synthetic peptide	*Carica papaya* L.	Hernandez M et al., 2007
	Gal/GalNAc lectin of *Entamoeba histolytica*	*Nicotiana Tabacum* L	Chebolu et al., 2007

Table 2. Pharmaceutical proteins expressed in transgenic plants (after year 2005).

Pharmaceutical protein	Host plant	Usage	References
Human acid *β*-glucosidase	*Nicotiana tabacum* L	Treatment of Gaucher disease	Reggi et al., 2005
Human serum albumin	*Oryza sativa* L.	Treatment of hypoalbuminemia or traumatic shock	Huang et al., 2005
Human lactoferrin	Rice	Iron absorption, immune system modulation and cellular growth promotion activity	Nandi et al., 2005
Human granulocyte-macrophage colony stimulating factor	*Oryza sativa*	Treating neutropenia	Sardana et al., 2007
Human basic fibroblast growth factor	*Glycine max*	Treatment of cardiovascular and neurodegenerative diseases	Ding et al., 2006
Human a-1-antitrypsin	*Solanum lycopersicum*	Maintaining protease antiprotease homeostasis	Agarwal et al., 2008
Human growth hormone	*Oryza sativa* L.	Treatment of dwarfism, bone fractures, skin burns, and bleeding ulcers	Kim et al., 2008[
Human α-L-iduronidase	*Nicotiana tabacum* L	Treatment of human lysosomal storage disorders	Kermode et al., 2007
Human epidermal growth factor	*Nicotiana tabacum* L	Treating trauma, burn, ulcer and corneal trauma	Bai et al., 2007
Glucagon-like peptide 1	*Oryza sativa* L.	Treating type II diabetes	Yasuda et al., 2005
Human fibroblast growth factor 8 isoform b	*Nicotiana tabacum* L.	targeting cancer therapy	Potula et al., 2008
Human bone morphogenetic protein2	*Nicotiana tabacum* L.	Bone tissue repair	Suo et al., 2006[
Mouse interleukin-12	*Lycopersicon esculentum*	Antitumor Immunity and antiviral Immunity	G-Ortega et al., 2005
Thrombomodulin	*Nicotiana tabacum* L	Treatment of thrombotic disorders	Schinkel et al., 2005

3) Choosing the suitable promoters

Employment of enhancer sequence, tissue-specific promoters and compound promoters offer an appropriate way for special and efficient expression of exogenous gene in plant tissue. With CAMV 35S promoter sequence, the activity of adjacent tissue- and organ-specific gene promoter are significantly affects that exhibit twofold to fivefold increase (Zheng et al., 2007). If we use duplication of a CaMV 35S promoter sequences, transcription efficiency of exogenous gene will display a huge improvement that tenfold higher than using CaMV 35S promoter sequence (Kay et al., 1987). Although CAMV 35S promoter is an efficient transcript regulators for foreign gene expression (Jani et al., 2002), it shows no specificity in neither plant tissue nor plant developmental stage, resulting in lower expression level sometimes (Smigocki and Owens, 1998). Fruit-specific promoter such as E8 promoter acted not only in an organ-specific, but also in a species-specific fashion in plant transformation. Thus it would be propitious to be flanked on foreign gene for targeting expression in fruits to achieve a high yield (He et al., 2008).

4) Usage of MAR sequences

Matrix attachment regions (MARs) are defined as DNA component that bind specifically to the nuclear matrix. The construction strategy of MARs is referred to making the exogenous gene independent transcription units by flanking MAR on either side, which could reduce the influence of adjacent elements. Many experiments have shown that MARs could enhance transgene expression and minimize transgene silencing (Tang and Wang, 2006). Mankin et al. (2003) has shown that the matrix attachment region (MAR) from the tobacco RB7 gene could significantly increased the average expression level for a variety of promoters. In addition, MAR sequences have ability to increase transformation frequencies and reduce variance of expression (Klaus et al., 2002).

5) Usage of virus vector

Compared with transgenic plant production system, plant

virus-based production system is more efficient for the iterative replication and fast iterative of viral vector in host cells. Foreign protein could reach a high accumulation level in a short time using plant viral expression systems. Otherwise, plant virus have a wide range of host species, so that any plant can be infected by viral vector has feasibility for mass production of heterologous proteins in abstracto (Matsuo et al., 2007).

High cost of downstream processing

In comparison with traditional pharmaceutical manufacturing, plant system offer a remarkable advantage that the upstream production cost is much lower. But extraction expense of gene expression product is high. Thus cost of downstream processing needs to be taken into consideration when upstream designing is carried out in production. Rational choice of host species is a modus operandi for reducing the cost. Banana is an ideal host for the production of edible vaccines as it offers advantages like digestibility and palatability by the infants, availability throughout the year in the tropics and subtropics where economical vaccines are required to immunize large segment of the population (Kumar et al., 2004). Kumar et al. (2005) gave the first report on the expression of HBsAg in transgenic banana fruits. Secondly, targeting to albuminous cells of edible seeds is applied to the expression of recombinant protein, which may enhance the stability of foreign protein. By using this method, it not only makes the storage and transport convenient but also simplifies the downstream processing and purification (Arcalis et al., 2004). Finally, plant cell suspension cultures may be a good choice for production of small-to-medium quantities of high-priced, high-purity, specialty recombinant proteins thanks to the greater control over the production environment, and simpler and cheaper downstream processing and purification (Kim et al., 2008).

Security problems

The increasing use of transgenic plants has raised wide attention about biosafety problems of genetically modified (GM) plants result from the safety of raw material and exogenous genes. Whether the genetically modified plants would give rise to significant risks to human health and environment has been the bone of contention. The following effects should be taken account, for instance, potential toxicity and allergenicity, compositional and nutritional characteristics, the influence of processing on the properties of the food or feed. Likewise, the escape of transgenes from GM plants to conventional plant may result in adverse effect to ecological environment (Anna and Frank, 2008). A consummate biosafety evaluation system is imperative with the development of genetic engineering.

PROSPECT

The limitations for the use of genetically modified plants will likely arise from our still somewhat unsophisticated knowledge of how plant gene expression is controlled and how various metabolic pathways within a plant interact and regulate themselves (Nilesh et al., 2004). Overcoming these bottlenecks will make improvement of the expression of target molecular. Pinpointing today's high demand of kinds of cost-effective recombinant proteins in therapy, diagnostic and other application, the use of plants as production factories is seen as an economically attractive alternative for the production. The lucubrating and exploitation of plant genetic technology will inevitably revolutionize the traditional industry, agriculture, pharmacy and other industries. With the isolation and cloning of new gene, and building of high-efficiency expression platform, a series of high and new plant products will continuously appears in the next 15 to 20 years.

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