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REVIEW

Production of recombinant human lactoferrin from transgenic plants

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Abstract

Molecular farming provides a powerful tool for low cost production of recombinant proteins with pharmaceutical value. The use of transgenic plants has been increasingly tested as alternative system for obtaining biologically active human lactoferrin in plants. Precise selection of plant species, transformation techniques and expression cassettes, in addition to conduction of detailed glycosylation and immunogenicity studies, serves as basis of obtaining safe recombinant human lactoferrin in high concentrations for the use of pharmacy. On the other hand, expression of antimicrobial protein lactoferrin in plants is a promising opportunity for crop quality improvement by increasing plant disease resistance.

Additional keywords: gene expression, molecular farming, plant-produced pharmaceutically important substances.

Introduction

Molecular farming is a new and reliable system for production of valuable therapeutic and industrial proteins in plants. Although recombinant proteins have been synthesized in a variety of bacterial, fungal and mammalian systems, using plants as bio-factories stimulated interest because higher economic benefit could be achieved (Horn *et al*. 2004). A major advantage of transgenic plants for molecular farming is low investment and operating costs of large scale production (Twyman *et al*. 2003, Chen *et al*. 2005). Other important advantages include lack of human or animal pathogens, oncogenic DNA sequences and endotoxins that could contaminate the final product. Furthermore, higher plants are able to synthesize proteins with correct folding, glycosylation and activity and, finally, plant cells can direct the protein of interest to environments that reduce its degradation (Horn *et al*. 2004).

According to Horn *et al*. (2004) proteins produced in plants for molecular farming purposes can be categorized into four groups: *1)* proteins used directly as pharmaceuticals along with those proteins used in the making of pharmaceuticals; *2)* enzymes such as hydrolases or oxido-reductases; *3)* monoclonal antibodies (IgA, IgG, IgM, secretory IgA, *etc*.) and antibody fragments; *4)* antigens for edible vaccines such as hepatitis B surface antigen (Mason *et al*. 1992, Sunil Kumar *et al.* 2007), cholera toxin B (Arakawa *et al.* 1998) or oncoproteins (Bříza *et al*. 2007).

A protein of increasing interest to molecular farming, which refers to the first group, is the iron-binding glycoprotein – lactoferrin (Lf). Lf is originally isolated from milk where it is found in high concentrations. It is normally detected in other exocrine secretions exposed to normal flora. Lf is a protein with a lot of physiological functions. Due to its iron-binding properties, Lf takes a part in iron absorbtion. It serves as a protective protein complementing the immunological defense system. One of the most important characteristics of Lf is the ability to act as natural anti-bacterial, antiviral, anti-fungal and anti-parasitic agent. Its bacteriostatic activity due to sequestration of the iron in the medium required for microbial metabolism whereas direct bactericidal and antifungal effect involve N-terminal antimicrobial domain (lactoferricin) which damages bacterial and fungal cell membranes (Yamauchi *et al*. 1993). The possible antiviral activity of Lf is due to inhibition of virus-cell fusion and viral entry into cells (van der Strate *et al*. 2001). Other functions of Lf include anti-tumor (Damiens *et al*. 1998), enzymatic (Kanyshkova *et al*. 2003, Hendrixson *et al*. 2003) and bone-growth regulation activities (Naot *et al*. 2005).

Because of the numerous important roles Lf plays, there is an increasing interest in establishing an expression system providing large amounts of biologically active recombinant protein.

Recombinant human Lf has been synthesized in

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Saccharomyces and *Aspergillus*. Probably because of low expression levels, the protein obtained in *Saccharomyces* is not commercially available (Liang *et al*. 1993). On the other hand, recombinant human Lf is successfully produced in *Aspergillus* at commercial scale (Ward *et al*. 1992, 1995) but extensive purification process is needed. Varieties of mammalian cell culture expression systems (Stowel *et al*. 1991, Van Berkel *et al*. 1995) as well as transgenic animals (Krimpenfort 1993, Van Berkel *et al*. 2005, Platenburg *et al*. 1994, Nuijens *et al*. 1997, Liu *et al*. 2005) are also being used in recombinant human Lf production. However, animal production systems have a number of disadvantages including long development time, expensive purification processes, logistics and potential for viral contamination.

Because of disadvantages of listed expression systems and aspiration for low cost large-scale production of recombinant human Lf scientists have turned attention to using plants as bio-factories. Human Lf is already successfully produced in various crops – tobacco (Liu *et al*. 1996, Salmon *et al*. 1998, Zhang *et al*. 1998), potato (Chong and Langridge 2000), tomato (Anzai *et al*. 2000, Lee *et al*. 2002), rice (Anzai *et al*. 2000, Nandi *et al*. 2002, Takase *et al*. 2005), maize (Samyn-Petit *et al*. 2001), barley (Kamenarova *et al*. 2007), ginseng (Kwon *et al*. 2003). High expression levels for the use of pharmacy can be obtained by using strong promoters and direction of protein accumulation in different parts of the plant – fruits, seeds, leaves and tubers. On the other hand expression of antimicrobial Lf in plants could be an opportunity for crop quality improvement by increasing its resistance to some diseases of significant economic importance.

Expression of recombinant human lactoferrin in transgenic tobacco plants and cell cultures

As a leafy, crop tobacco is suitable candidate for commercial production of recombinant human Lf because of the opportunity for high biomass production. Other advantages of tobacco include established transformation and expression technology, prolific seed production and the existence of a large-scale processing infrastructure (Twyman *et al*. 2003). Furthermore, tobacco is environmental safe because it is neither a food nor a feed crop.

Expression of human Lf gene under the control of 35S CaMV promoter was first reported in cultured tobacco (*Nicotiana tabacum*) cells, where expression levels of 1.8 % of total soluble protein have been achieved (Mitra and Zhang 1994). Although isolated protein seemed to be significantly smaller than full-length Lf protein, authors have shown that extracts from transgenic tobacco calli exhibited an antibacterial activity against four plant pathogenic strains bacteria. Full-length recombinant human Lf has been expressed in transgenic tobacco plants later (Liu *et al*. 1996, Salmon *et al*. 1998, Zhang *et al*. 1998). It was shown that recombinant protein confers a high level of resistance against cucumber mosaic virus (Liu *et al*. 1996) and significant delay of bacterial wilt symptoms (Zhang *et al*. 1998). On the other hand, although, similarity between recombinant and native protein molecules has been proved, establishing of tobacco produced Lf as commercially viable product for pharmaceutical or nutritional applications (Salmon *et al*. 1998) is restricted, because of the low expression levels obtained (0.1 - 0.3 % of total soluble protein). Due to presence of nicotine and other alkaloids, recombinant protein needs to be purified extensively before it could be considered safe for any food applications. There are, also, some limitations with regard to harvest, transport and storage, because protein stability is low in harvested material and must be processed immediately after harvest (Fischer *et al*. 2004).

An alternative technology for producing recombinant human Lf is using plant suspension culture. An advantage of this system is that recombinant proteins can be produced under certain conditions with large scale-up at low cost. (Fischer *et al*. 1999). In order to obtain higher extraction levels Choi *et al*. (2003) developed a system for expression of human Lf gene under the control of the strong stress-inducible peroxidase (SWPA2) promoter in tobacco cell suspension cultures. Over two fold more protein was produced than the amount generated by the 35S CaMV promoter (Mitra and Zhang 1994). The same system has been applied for production of higher levels of a recombinant human Lf (up to 3 % of total soluble protein) in ginseng (*Panax ginseng*) cell cultures (Kwon *et al*. 2003).

In order to use a recombinant protein for any pharmaceutical application, making a precise analysis of glycosylation pattern is very important. Post-translational biochemical processing of recombinant human Lf expressed in transgenic tobacco plants has already been characterized (Spik *et al*. 2000, Samyn-Petit *et al*. 2003). It has been shown that tobacco plants are able to produce protein with N-linked glycan having a core substituted by 2 N-acetylglucoseamine, 3 mannose and 1 fucose residues ($GluNAc_2(Fuc)Man_3$) as observed in human. The recombinant Lf produced in tobacco plants differs from native in some details including lower amount of galactose and GluNAc residues, lack of N-acetyl neuraminic acid residues, presence of xylose and replacement of $\alpha_{1\text{-}6}$ by $\alpha_{1\text{-}3}$ fucose in the N-linked glycan core. It has been demonstrated that differences in glycan composition does not affect cell binding properties of the recombinant protein (Salmon *et al*. 1998). Glycosylation pattern altered neither iron-binding properties nor bacteriostatic activity of recombinant human Lf expressed in tobacco, which shows that this system could be successfully used for crop quality improvement by increasing plant resistance against bacteria. Nevertheless, when the purpose of protein produced is clinical application, presence of xylose and $\alpha_{1\text{-}3}$ fucose in the

N-linked glycan core is of significant importance, because these two residues are highly immunogenic and might play role in allergenicity (Bardor *et al*. 2003, Chen *et al*. 2005). This might introduce some limitations to the pharmaceutical application of recombinant lactoferrin. That is why, making an effort to produce proteins with typical human glycan structures in plants by modification of glycosylation pathway *in vivo* is very important for obtaining reliable commercial product.

Expression of recombinant human lactoferrin in transgenic vegetable crops and cell cultures

Using fruits and vegetable crops as expression system for recombinant protein production is very advantageous because edible organs can be consumed as uncooked, unprocessed or partially-processed material.

Human Lf has been expressed in potato tubers (Chong and Langridge 2000). The protein obtained is stable during storage (Fischer *et al*. 2004) and could be used without expensive purification. Although anti-bacterial effect of recombinant protein against some human pathogenic bacterial strains was shown, low expression levels (0.01 - 0.1 % of total soluble protein) are making it unlikely as a commercially viable product. Another limitation is that potato contains solanin and needs to be cooked, so it is uncertain if the protein will retain its biological activity after boiling (Fischer *et al*. 2004, Lönnerdal *et al*. 2002).

Human Lf gene has, also, been expressed under the control of 35S CaMV promoter in sweet potato (*Ipomoea batatas*) cell suspension cultures (Min *et al*. 2006). This system was developed in order to produce recombinant therapeutic protein in a large scale with consequent application as a nutritional supplement. Nevertheless, low expression levels of 0.32 % of total extracted protein were achieved, compared with those obtained from tobacco and ginseng cell suspension cultures, using the SWPA2 promoter.

In order to enhance plant resistance to pathogens human Lf gene has been expressed in tomato (*Lycopersicon esculentum*) plants (Lee *et al*. 2002). Authors have reported that transgenic tomato lines exhibited resistance to bacterial wilt, which shows that this system could be successfully used for crop quality improvement.

Expression of recombinant human lactoferrin in cereals

Cereals are very attractive and preferred system for molecular farming because expression can be directed and protein is expressed as a storage protein in seeds. Seeds provide appropriate biochemical environment for stable accumulation of relatively high concentrations of the protein in a small compact biomass (Twyman *et al*. 2003, Stoger *et al*. 2005). Another advantage of cereal seeds is that they are free of toxic compounds in contrast to tobacco leaves and potato tubers, which makes them appropriate for application as food additives. Using seeds has, also, some bio-safety considerations – it limits exposure to non-target organisms such as microbes and herbivores (Stoger *et al*. 2005). Recombinant human Lf has already been successfully produced in transgenic rice, maize and barley plants.

Rice has a lot of advantages including high grain yields, capacity of rapid scale up and ease of transformation and manipulation (Stoger *et al*. 2005). Because of its low allergenicity and good nutritional value rice is one of the first "non-milk" foods introduced to infants so transgenic grains producing recombinant human Lf could be successfully used in infant formula without extensive purification (Nandi *et al*. 2002). On the other hand, constitutive expression of Lf gene in transgenic rice plants can be used for improving crop quality by increasing plant pathogen resistance (Takase *et al*. 2005).

In contrast to constitutive expression of human Lf gene in transgenic tobacco and potato plants, which provided low extraction levels, using seed directed expression under the control of rice gluteline promoter in rice, has provided obtaining recombinant protein in very high concentrations (Anzai *et al*. 2000, Nandi *et al*. 2002). Using this system, expression levels up to 20 % of total soluble protein [0.5 - 5 g kg^{-1}(rice seeds)] have been achieved (Nandi *et al*. 2002). Furthermore, authors have shown that the recombinant protein was biologically active and had properties similar to those of native human Lf. Amino acid sequence of recombinant and native protein was found to be identical and the specific sites were glycosylated. Rice derived Lf contained xylose and lacked sialic acid (Nandi *et al*. 2002, Lönnerdal 2002), which was unlikely to alter its anti-bacterial and iron-binding properties or to affect allergenicity. It was demonstrated that recombinant protein was capable to both bind and release iron and inhibited growth of some human pathogenic strains bacteria. Furthermore, authors reported that human Lf produced in rice was capable to bind specific Lf receptor, located in human infant small intestine (Kawakami *et al*. 1991). It was stable to *in vitro* protease digestion and breakdown by heat. Although these two characteristics show that rice-derived protein possibly would survive digestion in the infant gut and would retain its activity after some degree of heat treatment, they could be also a precondition for food allergies appearance. That is why, although demonstration that recombinant human Lf has identical biological activity with the native protein and very high

expression levels achieved, rice-based system should be subjected to further investigations.

Besides application as food additive, there is another benefit from expression of recombinant human Lf in rice plants – resistance to some plant pathogens. Takase *et al*. (2005) first reported constitutive expression of human Lf and its N-terminal peptide – lactoferricin in rice in order to investigate resistance against 3 classes of pathogens – bacterium, virus and fungus. While transgenic plants showed some resistance against bacterial seedling blight disease, they seemed to be sensitive to the viral and fungal pathogens tested and displayed typical disease symptoms.

Human Lf has also been expressed in maize in order to determine its N-glycosylation potential and use it as a model for monocotyledon expression system (Samyn-Petit *et al*. 2001). Authors have shown that recombinant maize Lf carried N-glycan structure similar to that obtained from tobacco and had all characteristics of a plant glycosylation pattern – presence of xylose and α_{1-3} fucose in the N-linked glycan core, absence of galactose and sialic acid residues and decrease of GlcNAc content. Differences in GlcNAc and galactose content in recombinant protein from maize and tobacco might due to difference in glycosylation in seeds and leaves, different subcellular localization or developmental stage of the plants (Samyn-Petit *et al*. 2003). Nevertheless, maize is advantageous and preferred system for pharmaceutical proteins production because of the higher annual grain yield and shorter generation interval when compared to rice and barley and ease of *in vitro* manipulation and transformation (Stoger *et al*. 2005). That is why, although difference in N-glycan structure, authors turned their attention to further engineering of maize N-glycosylation in order to produce safe and efficient pharmaceutical protein.

Alfalfa – a reliable candidate for development a new system for expression of recombinant human lactoferrin

Alfalfa (*Medicago sativa*), a perennial forage crop, is a suitable candidate for development of a new and efficient system for recombinant Lf production, because of its favourable agronomic characteristics. It is harvested several times per year which provides short-term high biomass production. As a legume crop, alfalfa benefits from its ability to fix atmospheric nitrogen which reduces need of fertilization. Other advantages of alfalfa include ease of transformation and clonal propagation which is important for rapid amplification of transgenic plants obtained. These characteristics provide an opportunity for low cost large-scale recombinant protein production. Using this fodder crop as an expression system, also, gives a chance for obtaining high quality recombinant human Lf for the use of pharmacy. Lack of toxic compounds makes alfalfa appropriate candidate, because no extensive purification process is needed. It has been shown that recombinant C5-1 antibody produced in transgenic alfalfa plants exhibited homogenous N-glycosylation (Bardor *et al*. 2003, D'Aoust *et al*. 2004), which ensures batch-to-batch reproducibility and is suitable for *in vitro* N-glycan modification. It means that using alfalfa expression system for recombinant protein production could solve problems, relevant to plant N-glycosylation pattern and potential allergenicity of the product. Moreover, it has been shown that recombinant proteins were highly stable in alfalfa protein extract and could be stored for long time without detectable degradation, because of weak proteolytic activity (Busse *et al*. 2001).

Expression of human Lf in transgenic alfalfa plants could be beneficial not only for obtaining high quality recombinant protein for clinical application but also for crop quality improvement. Alfalfa is main forage plant because of its nutritional value – high protein, mineral and vitamin content and preferred feed for different classes of livestock. That is why there is an interest in improving alfalfa quality and yields by expression antimicrobial protein Lf, which confers resistance to some plant pathogens.

Conclusion

Lf is a multifunctional glycoprotein, which takes a part in a large number of important physiological processes. Using transgenic plants as bio-factories is a new approach to recombinant Lf production. High expression levels could be achieved by precise selection of plant species for molecular farming and using appropriate promoter for constitutive or organ directed expression. Furthermore, a lot of efforts are made for developing strategies for *in vivo* modification of plant glycosylation pathway in order to overcome limitations concerning plant N-glycan structure. It has been shown that expression of human Lf in transgenic plants confers resistance to plant pathogens, which means that this system could be also successfully used for crop quality improvement.

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