

# Recombinant Expression Systems for Allergen Vaccines

Mohan B. Singh\* and Prem L. Bhalla

*Plant Molecular Biology and Biotechnology Laboratory, Australian Research Council Centre of Excellence for Integrative Legume Research, Institute of Land and Food Resources, The University of Melbourne, Parkville, Vic 3010, Australia*

**Abstract:** Allergen immunotherapy of future is likely to be based on allergy vaccines that contain engineered allergens modified to abolish or substantially reduce their IgE-binding activity in order to remove the risk of unwanted anaphylactic responses.

The development of efficient systems for the production of recombinant allergens in sufficient quantities is requirement for establishing use of engineered allergens as components of allergy vaccines. This review outlines relative advantages and disadvantages of various heterologous systems for production of recombinant allergens. Microbial systems are most convenient and cost effective platforms for the production of recombinant allergens. However, lack of post-translational processing implies that some allergens have to be expressed in eukaryotic systems for proper folding and post-translational modifications such as glycosylation. Yeast systems can yield high levels of recombinant allergens but often are associated with hyper- glycosylation problems. Mammalian cell culture systems offer suitable post –translational modifications but are nearly hundred fold more expensive than microbial systems. The use of plants as bio-factories for production of recombinant allergens is emerging as a very attractive option as plants-based production system offer several advantages over other expression systems such as post translational processing of proteins, low production costs, scale up ability and enhanced safety due to absence of animal or human pathogens.

## INTRODUCTION

The development of recombinant allergen based allergy vaccines is an emerging strategy for the diagnosis and immunotherapy of type 1 allergic disorders [1-4]. Efficient systems for the production of recombinant allergens are prerequisite for establishing their use in future clinical applications. Recombinant DNA techniques have facilitated the development of new principles for design and production of allergy therapeutics. The target protein allergen can be made safer by generation of hypoallergenic forms and the immunogenic properties of these hypoallergenic forms can be significantly enhanced by genetic modifications such as addition of immunopotentiating tags or production of hybrid molecules. In addition, recombinant DNA technology offers a number of strategies for the construction of genes encoding allergens. Building up genes *de novo* using synthetic oligonucleotides can be very useful if the GC content of the gene to be expressed differs significantly from that of host selected for expression. Adjustment of codon frequency of the allergen to match that of the heterologous expression system is often a prerequisite to obtain enhanced expression levels. Codon optimisation can now be readily achieved by constructing synthetic genes based on conversion of amino acid sequences into a DNA sequence with codon usage similar to host organism. Though microbial systems are considered to be convenient platforms for the production of recombinant allergens the final choice of expression system is dependent upon the nature of allergen to be produced. Therefore, this review will focus on different expression systems available for recombinant allergen production.

Fig. 1 outlines steps involved while Table 1 provides selected examples of various recombinant allergens produced in different host systems.

## BACTERIAL EXPRESSION SYSTEM

The ideal system for the expression of recombinant allergens for their application in development of allergy diagnostic or therapeutic reagents has to be essentially the same as is used to identify these proteins as allergens. Since most of the protein allergens have been identified through screening of cDNA expression libraries for IgE reactive recombinant fusion proteins, large scale bacterial cultures simply represent scale up of the same technology that was used to identify allergens in the first place.

*Escherichia coli* is one of the most widely used bacterial hosts for the production of recombinant proteins because of easy transformation and its rapid growth in an inexpensive, easy-to-prepare growth medium. *E. coli* thus offers a convenient and versatile system for the production of recombinant allergens. However, *E. coli* is a prokaryote and lacks cellular machinery present in eukaryotes that is responsible for post-translational processing and modifications. The major shortcoming of bacterial expression system is frequent failure of recombinant proteins to fold in native biologically active state. Expression of recombinant protein in *E. coli* often results in the production of insoluble protein forming inclusion bodies, which require solubilization with strong chaotropic agents followed by gradual refolding to obtain functionally active protein [5]. For instance cypress major allergen, Cup a 1.02 expressed as a N-terminal 6xHis-tagged protein in *E. coli* required such solubilization and refolding to recover IgE reactive form of allergen [5].

\*Address correspondence to this author at the Institute of Land and Food Resources, The University of Melbourne, Parkville, Vic 3010, Australia; Tel/Fax: +61 3 8344 5051; E-mail: mohan@unimelb.edu.au

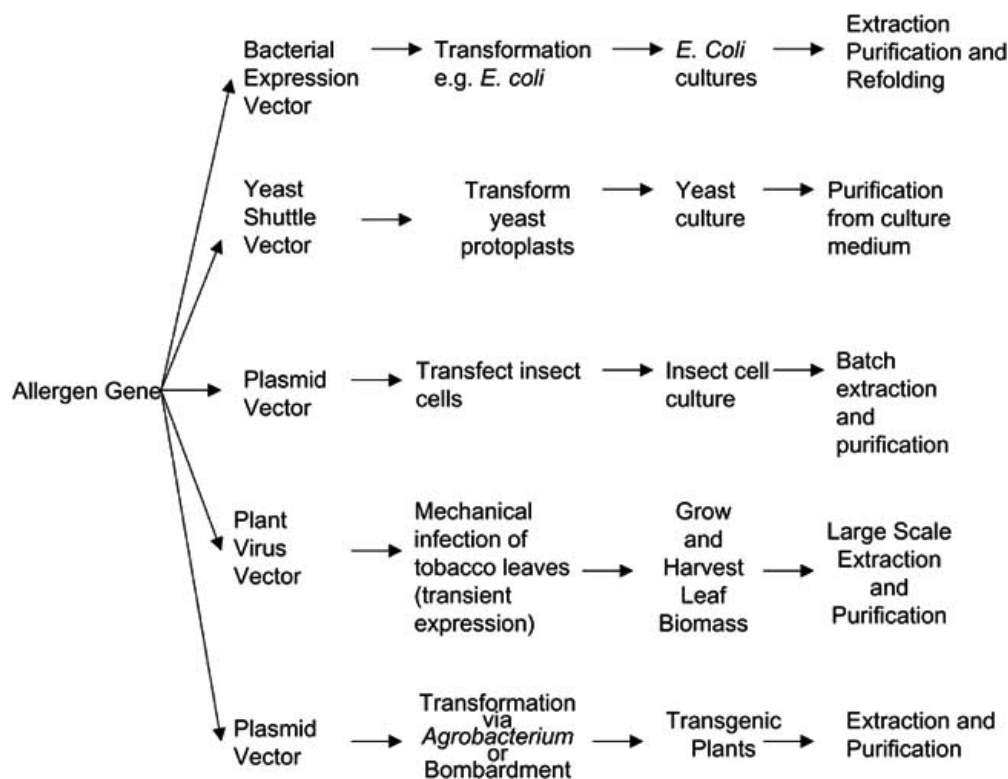


**Fig. (1).** Schematic depiction of steps involved in recombinant allergen expression using different host systems.

**Table 1.** Examples of Recombinant Allergen Expression in Various Host Systems

| Host System            | Allergen Source                              | Allergen           | Outcome                                                                                                                             | Ref. |
|------------------------|----------------------------------------------|--------------------|-------------------------------------------------------------------------------------------------------------------------------------|------|
| <i>E. coli</i>         | Peanuts                                      | Ara h 2            | Expressed as His tag protein in <i>E.coli</i> (origami) strain, properly folded and IgE reactive similar to natural allergen        | [8]  |
|                        | <i>Cupressus</i> pollen                      | Cup a 1            | RCup a1 accumulated in inclusion bodies; successful refolding resulted in allergen with IgE reactivity similar to natural allergen  | [5]  |
| <i>Pichia pastoris</i> | <i>Plantanus</i> pollen                      | Pla 11             | Secreted in media at 20mg/litre, proper folding shown by CD spectra, IgE reactive                                                   | [15] |
|                        | Olive pollen                                 | Ole e 1            | rOle e 1 secretion in media at 60 mg/litre: IgE reactive and immunologically equivalent to natural form                             | [12] |
|                        | <i>Dactylis</i> pollen                       | Dac g 5            | rDac g 5 secreted in media at 15 mg/litre : hyperglycosylation, IgE reactive                                                        | [14] |
|                        | Cockroach                                    | Bla g 4            | rBla g 4 secreted at the concentration of 50 mg/litre: produced 23 kD glycosylated allergen: IgE reactive                           | [11] |
|                        | Natural rubber latex                         | Hev b 7            | rHev b 7 secreted at 10 mg/litre culture medium: immunological equivalence to natural Hev b 7                                       | [20] |
| Mammalian Cells (CHO)  | House dust mite ( <i>Blomia tropicalis</i> ) | Blo t 5            | Codon optimisation led to 13 mg/litre production of rBlo t 5 in a CHO-K Clone; high correlation of IgE reactivity with natural form | [30] |
|                        | House dust mite                              | Der p 1            | Codon optimised synthetic Pro Der p1 gene transfected in CHO cells produced up to 30 mg/litre Pro Der p1; rProDer p1                | [29] |
| Transgenic Plants      | Japanese cedar pollen                        | Cry j 1            | rCry j 1 produced in transgenic rice seeds, allergen accumulation in protein bodies                                                 | [42] |
|                        | Birch pollen                                 | Bet v 1            | Infected <i>Nicotiana benthamiana</i> leaves produced 200microg/g leaf material with IgE binding comparable to natural allergen     | [46] |
| Plant virus system     | Natural rubber latex                         | Hev B 1<br>Hev B 3 | Properly folded and IgE reactive rubber latex allergens produced in <i>Nicotiana benthamiana</i>                                    | [51] |

Co-production of chaperone proteins in bacteria has been shown to assist in proper folding and avoiding deposition in inclusion bodies [6]. However, the selection of suitable chaperone is an empirical process and this strategy has not

been applied to production of recombinant allergens in *E. coli*. Another disadvantage of bacterial system is the inability of *E. coli* to secrete the protein in extra-cellular medium. Isolation of recombinant protein requires cell disruption thus

also releasing many contaminants thereby increasing purification costs. Codon usage is known to present a potential impediment to high-level gene expression in *E. coli*. It is an established fact that "rare" codons, depending on their frequency and context, can have an adverse effect on protein levels. Usually, this problem can be alleviated by modification of the relevant codons.

Some of the successful strategies to enhance the quality and yield of recombinant proteins expressed in bacteria have focussed on genetic improvements such as design of better expression vectors and the improvement of bacterial strains by deletion of protease genes or genetic engineering for overexpression of chaperones or rare-codon tRNAs [7]. Some of new bacterial host strains facilitate formation of disulfide bonds in reducing environment of the cytoplasm and offer higher protein yields by minimizing proteolytic degradation. Properly folded pea nut allergen, Ara h 2 was produced by expression in a modified *E. coli* strain Origami (DE3) that contains oxidizing cytoplasm allowing the formation of disulfide bonds [8].

## YEAST BASED EXPRESSION

Yeast is an attractive host for the production of recombinant allergens. Being eukaryotes, yeast cells can carry out many of the post-translational folding, processing and modifications required to produce "authentic" and biologically active allergen proteins. They retain the advantages of a unicellular microorganism with respect to rapid growth and ease of genetic manipulation therefore can be grown to high density for high amounts of recombinant protein production. Moreover, yeast system can perform many eukaryotic post-translational modifications, such as glycosylation, disulfide bond formation and proteolytic processing.

Two most commonly used yeast strains are *Pichia pastoris* and *Saccharomyces cerevisiae*. *Pichia pastoris*, methylotrophic yeast, with ability to grow on methanol as a sole carbon source offers one of the most successful system for the production of a variety of recombinant proteins from milligram to gram quantities [9]. Techniques required to genetically transform *Pichia* are relatively simple and recombinant proteins can be produced at a high amount either intracellularly or as extracellular secreted protein in the culture medium [10]. Large amount secretion of recombinant proteins into an almost protein-free medium makes scaling up and downstream purification much easier task. Moreover, *Pichia* does not have the endotoxin problem associated with bacteria. Since the proteins produced in *P. pastoris* are typically folded correctly and secreted into the medium, the fermentation of genetically engineered *P. pastoris* provides an excellent alternative to *E. coli* expression systems. For instance, expression of cockroach allergen, Bla g 4 in *E. coli* yielded only 0.25 mg/L culture whereas 50mg/L of secreted allergen protein was obtained in *Pichia pastoris* [11].

An important aspect of recombinant allergen expressed in yeast is the feature of post-translational processing and modification processes specific to yeast. N-and O-linked glycosylation patterns in yeast may prove to be different from those in the native host. For example, yeast adds

mannose units to threonine or serine residues, while higher eukaryotes prefer sialic acid O-linked side chains. Such differences may affect the folding, stability, activity and immunogenicity of allergens produced in yeast. By contrast, N-linked glycosylation in yeast largely resembles that of higher eukaryotes. Glycoprotein production in yeast expression system has been reported to contain a mixture of forms with a variable number of mannose residues [12]. However, this variation in glycosylation does not seem to affect immunological properties of recombinant allergens. In case of Pla 11, CD spectra of recombinant versus natural allergen showed that variations in glycosylation have no effect on protein folding pattern. Cloning of major house mite allergen in *Pichia* system resulted in secretion of 40-60 kDa hyperglycosylated pro-protein which showed autocatalytic maturation to mature form [13]. However spontaneous maturation of r Der p 1 was shown to be inhibited by hyperglycosylation [14]. Enzymatic deglycosylation of rpro-Der p1 by treatment with endoglycosidase H was required to achieve recovery of mature Der p 1 protein [14].

There are number of recent studies on use of yeast expression system. Expression of Pla 11, a major allergen from English plantain (*Plantago lanceolata*) pollen in *Pichia pastoris* system resulted in secretion of the recombinant allergen in the culture medium at the level of 20 mg per litre [15]. The secreted allergen was a mixture of non-glycosylated and glycosylated forms. Ole e 6, a low molecular weight (5.8kDa) allergen of olive pollen has been efficiently expressed in *Pichia pastoris* [16]. However, about 50% of the recombinant protein was shown to have one of the methionine in oxidized state resulting in the alteration of electrophoretic mobility. Major allergens of Lilac (*Ligustrum vulgare*) and privet pollen expressed in *Pichia pastoris* retained their natural conformation comparable to that of natural allergens [17]. Expression of major house dust mite allergen, Der f1 resulted in the production of biologically active form of allergen that showed IgE reactivity and cysteine protease activity comparable to its natural counterpart [18]. Natural rubber latex allergen, Hev b 7, has been expressed in *Pichia pastoris* at 10 mg/l culture supernatant. Both natural and recombinant forms of this allergen showed IgE reactivity in the latex-allergic patients [19, 20].

One of the main disadvantages of yeast expression systems is hyper-glycosylation of proteins. Sometimes more than 100 mannose residues are attached as compared to normal 8-13 molecules. An emerging field is the artificial *in vivo* glycoengineering of *Pichia pastoris* strains to develop practical tools for designer glycosylation pathways [21]. Such genetic engineering of yeast to produce different kind of glycosylated proteins [21] would be valuable for expression of recombinant allergens.

## EXPRESSION IN INSECT CELL CULTURE

Insect cells used in combination with baculovirus expression vector system are gaining widespread use as a platform for recombinant protein production. These expression systems are based on the ability of baculoviruses to infect insect cells. Baculoviruses infect primarily insects with a narrow host range. *Autographa californica*, the most

commonly used baculovirus for protein expression, infects only 2 lepidopteran (moth) families in nature. The heterologous proteins using baculoviruses as vectors can be produced both in insect cells and larvae [22]. The procedure for baculovirus based recombinant expression involves cloning of target gene in a bacterial shuttle vector, which is then propagated in *E. coli* cells. Insect cells infected with AcMNPV are transfected with shuttle vector followed by identification of cells in which homologous recombination has taken place. Recombinant virus is then isolated and used to infect new insect cells followed by protein harvesting 36-48 h post-infection. Baculovirus system kills the cells so continuous production is not possible making this system suitable only for batch production of recombinant proteins.

As with other eukaryotic expression systems, baculovirus expression of foreign proteins allows post-translational modifications. The insect cell cytoplasmic environment allows proper folding and S-S bond formation. Single polypeptide, dimeric and trimeric proteins have been expressed in baculoviruses. Moreover, expression of heterologous proteins is under the control of a strong polyhedrin promoter, allowing up to 30% of the total cell protein to be recombinant protein.

Baculovirus-insect cell expression system is widely used as a platform technology for production of recombinant proteins [23]. However, this system is typically unable to produce recombinant glycoproteins with authentic oligosaccharide side chains.

Major house dust mite allergen rDer f expressed in insect cells showed only 20% of IgE reactivity of the natural counterpart [24]. Autocatalytic removal of pro sequence by incubation in an acidic pH led to increase in IgE reactivity similar to natural allergen [24]. Bee venom allergen hyaluronidase when expressed in *E. coli* showed 20-30% of enzymatic activity and IgE reactivity of natural allergen. However, same allergen produced in baculovirus-infected insect cells showed biological activity and immunoreactivity comparable to natural allergen [25]. Recombinant expression of bee venom hyaluronidase in insect cells yielded high levels of pure protein thus allowing determination of its crystal structure [26].

Some allergens are composed of two polypeptide chains and therefore both subunits have to be expressed at the same time and in stoichiometric amounts. These requirements can be met by simultaneous expression of two genes in one plasmid. The major domestic cat allergen, Fel d 1, has proven difficult to express in immunoreactive form in bacterial systems because this allergen is a heterodimer encoded by two different genes. However, co-expression of two Fel d 1 chains in baculovirus system led to production of mature protein with comparable immunoreactivities to nFel d 1 [27]. Recently, baculovirus expression system in insect cells has been successfully used to obtain a high-level expression of hypoallergenic forms of natural rubber latex allergen, Hevein [28].

## MAMMALIAN CELL CULTURE EXPRESSION SYSTEM

Mammalian cells have the cellular machinery capable of extensive posttranslational modifications and processing.

Expression of recombinant (rec) ProDer p 1, a recombinant precursor form of a major house dust mite allergen, Der p 1, in Chinese hamster ovary (CHO) cells resulted in production of up to 30 mg of recProDer p 1 per litre of the culture [29]. A synthetic gene encoding ProDer p 1 was designed on the basis of codon usage frequently found in highly expressed human genes. High level of expression (13 mg/litre) of codon optimised synthetic gene for house dust mite, *Blomia tropicalis*, in Chinese hamster ovary (CHO)-K1 cells has been reported [30]. However, high-level expression of a recombinant protein in Chinese hamster ovary cells typically requires laborious and time-consuming procedure of stepwise gene amplification [31]. Mammalian cell cultures also require expensive media. Use of mammalian cells is also associated with safety concerns from potential contamination with mammalian viruses and other pathogens.

## PLANT BASED EXPRESSION SYSTEMS

Plant systems for the production of recombinant proteins have number of clear advantages such as appropriate post-translational modifications, flexibility to rapidly scale up or down production, reduced input costs and absence of potential to harbour human pathogens. Transgenic plants can be engineered to accumulate proteins in their seeds or grains or leaves making them low cost bio-factories since fermentation bioreactors are essential for microbial and animal cell cultures.

Virtually, any plant (genetically transformable) can be used to produce recombinant proteins. Tobacco, alfalfa, and cereal crops like corn, rice, and barley are among the plants tested as biofactories for biopharmaceuticals. The strategies for designing expression cassettes for various plant based expression systems are summarized in Table 2.

Each plant system has unique advantages and disadvantages. For crop plants, there is a well-established infrastructure for their cultivation, harvest, and storage. Cereal grains and oilseeds can be stored for long periods without loss of protein or its biological activity. Legumes such as soybeans and alfalfa, can fix nitrogen from the atmosphere and thus require less chemical input for growth. It is possible to engineer plant that produces recombinant allergen in its seeds, leaves or fruits. Such plants can be grown in large quantities in field conditions to produce kilogram quantities of recombinant allergen proteins. Alfalfa leaves are particularly suitable for recombinant allergen production since leaves yield up to 20% total protein on a dry weight basis [32].

A major concern with using plants as production platforms for recombinant allergen production is maintaining their isolation from food production chains. For large field scale production, containment measures such as isolation distances or male sterility are necessary. For small to medium scale production plants can be grown in a contained situations such as growth cabinets or glass houses.

Recent advances in plant-based expression include use of a moss, *Physcomiterella patens*, as a host system. Mosses are eukaryotes capable of performing post-translational modifications of proteins. *Physcomiterella* is a photoautotroph that can grow in bioreactors [33].

**Table 2. Gene Construct Designs for Optimal Expression of Allergen Vaccines in Plant Systems**

| No. | Object                                               | Approach and Outcome                                                                                                                                                                                                 | Ref.         |
|-----|------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------|
| 1   | Codon frequency optimisation                         | Prepare synthetic genes with codon frequencies preferred by plants                                                                                                                                                   | [52, 53, 54] |
| 2   | Sequence optimisation                                | Design genes with base composition suitable for plant expression and optimise secondary structure to minimize hairpins in order to achieve efficient translation                                                     | [55]         |
| 3   | Use of tissue specific promoter                      | Consider use of either constitutive, tissue specific or inducible promoter                                                                                                                                           | [42, 43]     |
| 4   | Targeting of protein to endoplasmic reticulum        | Add endoplasmic reticulum retention sequence such as KDEL to the C terminus of the allergen                                                                                                                          | [56]         |
| 5   | Accumulation of seed protein bodies                  | Use of transit peptide sequences to achieve accumulation of high levels of recombinant protein in protein bodies                                                                                                     | [42, 43]     |
| 6   | Use of ubiquitin fusion                              | Express proteins as C-terminal translational fusions with ubiquitin to augment protein accumulation                                                                                                                  | [60]         |
| 7   | Targeting of proteins to plastids                    | Add a transit peptide sequence to the N terminus of allergen gene to direct transport and accumulation in chloroplasts, amyloplasts or other plastids                                                                | [57]         |
| 8   | Integration of target gene in the chloroplast genome | Introduce target protein encoding gene into chloroplasts by microprojectile bombardment for integration into its genome by homologous recombination: resulting in protein synthesis and accumulation in chloroplasts | [58, 59]     |
| 9   | Use of plant viral vectors                           | Infect <i>Nicotiana benthamiana</i> leaves with engineered TMV virus carrying target gene to achieve high level production in leaves without stable genome transformation                                            | [47, 48]     |

Recombinant proteins are secreted in a simple medium that greatly facilitates downstream processing [34]. Being a contained bioreactor system, this allows proteins to be produced without concerns of releasing transgenes.

Plant chloroplast transformation has been demonstrated as an environmentally friendly approach that minimizes transfer of genes to related weeds and crops [35, 36]. Chloroplast genomes can be genetically engineered to produce recombinant proteins. Moreover, transformation of chloroplasts allows the introduction of several hundred copies of foreign genes per plant cell leading to higher amount of recombinant protein production. Often 100 -fold higher level of expression can be achieved as compared to when foreign protein is produced by introducing gene in nuclear genome [37-39].

Yield of the recombinant protein can vary from 0.1% to 10% or more, depending on the plant, intracellular location of the protein expression and the type of expression system employed. Plant produced recombinant proteins are particularly attractive as plants are free of human or animal diseases, reducing screening costs for viruses and bacterial toxins. Recombinant proteins produced in plants have been shown to induce appropriate immune responses [40, 41].

Using stable plant transformation approach Okada *et al.* [42] expressed recombinant Japanese cedar pollen allergen, Cry j 1 in rice grains by directing expression in seeds using seed storage protein promoter. Targeting Cry j 1 expression in the protein bodies of developing seed resulted in accumulation of the recombinant protein at 15-50 fold higher than in cytoplasm expression system. In addition, expression of Cry j 1 as two fragments, N-terminal and C-terminal halves resulted in generating hypoallergens in rice seeds for potential use in oral immunotherapy. Sunflower seed albumin (SSA), a potential allergen has been expressed in narrow leaf lupin, (*Lupinus angustifolius*) seeds [43]. In this

study a legume seed storage promoter was used to target the expression and accumulation of SSA in the lupin seeds. Significant levels of SSA reaching 5% of total extractable seed protein were recorded [43]. Oral immunotherapy using meal of SSA-lupin seeds in mouse model system showed dramatic suppression of SSA-specific experimental asthma [44].

Recently, plant viruses have also emerged as tools for production of large amounts of recombinant proteins in plants. Plant RNA viruses are engineered to carry and replicate foreign genes in susceptible host plants. These RNA viruses provide a transient expression system as foreign genes and viral genomic RNA sequences do not integrate in to the plant genome. These sequences are amplified by viral replicase in cytoplasm of plant cell. After replication, the RNA is then translated by plant cell translational machinery. Therefore, foreign proteins are subjected to post translational modifications in the same way as proteins produced by stable transgenic plant cells.

Tobacco mosaic virus (TMV) is a single stranded RNA virus that produces large quantities of RNA and protein on plant cell infection making TMV an attractive candidate for expression of recombinant allergens in plants. TMV based expression of foreign proteins was first tested for expression of bacterial proteins in *Nicotiana benthamiana* [45]. First published report of TMV directed expression of allergen was on the production of major birch pollen allergen, Bet v1 in *Nicotiana benthamiana* [46]. The recombinant allergen representing 0.6% of the total soluble proteins in leaves was structurally and immunologically equivalent to natural Bet v1 purified from birch pollen. Apple fruit allergen, Mal d1 has been expressed in *Nicotiana benthamiana* plants by using tobacco mosaic viral vector. Transfected *N. benthamiana* plants showed accumulation of Mal d 1 at 2% of the total soluble leaf protein [47]. Recombinant Mal d 2 produced in

*N. benthamiana* displayed the ability to bind IgE from apple-allergic individuals equivalent to natural Mal d 2.

Recently, a novel viral engineering and expression system has been reported [48]. This system is claimed to be very fast and is reported to accumulate recombinant protein up to 80% of total soluble protein. This modified viral system provides most promising strategy for scalable production of recombinant allergen proteins. These new developments make plant viral expression system as one of the most useful tool for expression of recombinant allergens in plants.

## CONCLUSIONS

Current advances in the production of engineered hypoallergenic forms of major allergens are likely to lead in generation of safer vaccines for allergy immunotherapy. In order to ensure the utilization of such immunotherapy reagents in future, it will be necessary to develop efficient systems for their production. Availability of well-known structurally related allergens would also be useful in improved diagnosis and determination of cross reactivity patterns. Bacterial expression systems are convenient for allergens that do not need post-translational modifications. However to be biologically active some allergens may need to be expressed in eukaryotic systems. There are emerging strategies such as the use of fusion proteins to facilitate the downstream purification of recombinant proteins and the fusion of target protein immunogens with carrier proteins can increase both the half life and immunogenicity of the proteins [49]. Overall, of all the expression systems plants hold the best promise for expression of recombinant allergens for their use in allergy vaccines [50]. Rather low levels of expression hampered earlier use of plants for expression of recombinant allergens. However, recent developments such as chloroplast expression and gene amplification using viral vectors have shown that the promise of large scale "pharming" of modified allergens for their application in immunotherapy will soon be realized.

## REFERENCES

- Valenta, R.; Kraft, D. *Curr. Opin. Immunol.*, **1995**, 7, 751.
- Kraft, D.; Ferreira, F.; Ebner, C.; Valenta, R.; Breiteneder, H.; Susani, M.; Breitenbach, M.; Scheiner, O. *Allergy*, **1998**, 53, 62.
- Bhalla, P.L. *Exp. Rev. Vaccines*, **2003**, 2, 75.
- Bhalla, P.L.; Singh, M.B. *Curr. Opin. Allergy Clin. Immunol.*, **2004**, 4, 569.
- Rea, G.; Iacovacci, P.; Ferrante, P.; Zelli, M.; Brunetto, B.; Lamba, D.; Boffi, A.; Pini, C.; Federico, R. *Protein Expr. Purif.*, **2004**, 37, 419.
- Hoffmann, F.; Rinas, U. *Adv. Biochem. Eng. Biotechnol.*, **2004**, 89, 143.
- Jonasson, P.; Liljeqvist, S.; Nygren, P.A.; Stahl, S. *Biotechnol. Appl. Biochem.*, **2002**, 35, 91.
- Lehmann, K.; Hoffmann, S.; Neudecker, P.; Suhr, M.; Becker, W.M.; Rosch, P. *Protein Expr. Purif.*, **2003**, 31, 250.
- Cereghino, J.L.; Cregg, J.M. *FEMS Microbiol. Rev.*, **2000**, 24, 45.
- Yokoyama, S. *Curr. Opin. Chem. Biol.*, **2003**, 7, 39.
- Vailes, L.D.; Kinter, M.T.; Arruda, L.K.; Chapman, M.D. *J. Allergy Clin. Immunol.*, **1998**, 101, 274.
- Huecas, S.; Villalba, M.; Gonzalez, E.; Martinez-Ruiz, A.; Rodriguez, R. *Eur. J. Biochem.*, **1999**, 261, 539.
- Jacquet, A.; Magi, M.; Petry, H.; Bollen, A. *Clin. Exp. Allergy*, **2002**, 32, 1048.
- van Oort, E.; de Heer, P.G.; van Leeuwen, W.A.; Derksen, N.I.; Muller, M.; Huveneers, S.; Aalberse, R.C.; van Ree, R. *Eur. J. Biochem.*, **2002**, 269, 671.
- Calabozo, B.; Diaz-Perales, A.; Salcedo, G.; Barber, D.; Polo, F. *Biochem. J.*, **2003**, 372, 889.
- Barral, P.; Tejera, M.L.; Trevino, M.A.; Batanero, E.; Villalba, M.; Bruix, M.; Rodriguez, R. *Protein Expr. Purif.*, **2004**, 37, 336.
- Gonzalez, E.; Villalba, M.; Rodriguez, R. *Clin. Exp. Allergy*, **2001**, 31, 313.
- Yasuhara, T.; Takai, T.; Yuuki, T.; Okudaira, H.; Okumura, Y. *Clin. Exp. Allergy*, **2001**, 31, 116.
- Sowka, S.; Wagner, S.; Krebitz, M.; Arijia-Mad-Arif, S.; Yusof, F.; Kinaciyan, T.; Brehler, R.; Scheiner, O.; Breiteneder, H. *Eur. J. Biochem.*, **1998**, 255, 213.
- Breiteneder, H.; Sowka, S.; Wagner, S.; Krebitz, M.; Hafner, C.; Kinaciyan, T.; Yeang, H.Y.; Scheiner, O. *Int. Arch. Allergy Immunol.*, **1999**, 118, 309.
- Bobrowicz, P.; Davidson, R.C.; Li, H.; Potgieter, T.I.; Nett, J.H.; Hamilton, S.R.; Stadheim, T.A.; Miele, R.G.; Bobrowicz, B.; Mitchell, T.; Rausch, S.; Renfer, E.; Wildt, S. *Glycobiology*, **2004**, 14, 757.
- Geisse, S.; Gram, H.; Kleuser, B.; Kocher, H.P. *Protein Expr. Purif.*, **1996**, 8, 271.
- Ikononou, L.; Schneider, Y.J.; Agathos, S.N. *Appl. Microbiol. Biotechnol.*, **2003**, 62, 1.
- Shoji, H.; Hanawa, M.; Shibuya, I.; Hirai, M.; Yasuhara, T.; Okumura, Y.; Yamakawa, H. *Biosci. Biotechnol. Biochem.*, **1996**, 60, 621.
- Soldatova, L.N.; Cramer, R.; Gmachl, M.; Kemeny, D.M.; Schmidt, M.; Weber, M.; Mueller, U.R. *J. Allergy Clin. Immunol.*, **1998**, 101, 691.
- Markovic-Housley, Z.; Miglierini, G.; Soldatova, L.; Rizkallah, P.J.; Muller, U.; Schirmer, T. *Struct. Fold. Des.*, **2000**, 8, 1025.
- Vailes, L.D.; Sun, A.W.; Ichikawa, K.; Wu, Z.; Sulahian, T.H.; Chapman, M.D.; Guyre, P.M. *J. Allergy Clin. Immunol.*, **2002**, 110, 757.
- Karisola, P.; Mikkola, J.; Kalkkinen, N.; Airene, K.J.; Laitinen, O.H.; Repo, S.; Pentikainen, O.T.; Reunala, T.; Turjanmaa, K.; Johnson, M.S.; Palosuo, T.; Kulomaa, M.S.; Alenius, H. *J. Immunol.*, **2004**, 172, 2621.
- Massaer, M.; Mazzu, P.; Haumont, M.; Magi, M.; Daminet, V.; Bollen, A.; Jacquet, A. *Int. Arch. Allergy Immunol.*, **2001**, 125, 32.
- Lim, L.H.; Li, H.Y.; Cheong, N.; Lee, B.W.; Chua, K.Y. *Biochem. Biophys. Res. Commun.*, **2004**, 316, 991.
- Running Deer, J.; Allison, D.S. *Biotechnol. Prog.*, **2004**, 20, 880.
- Larrick, J.W.; Yu, L.; Naftzager, C.; Jaiswal, S.; Wycoff, K. *Biomol. Eng.*, **2001**, 18, 87.
- Decker, E.L.; Reski, R. *Curr. Opin. Plant Biol.*, **2004**, 7, 166.
- Hohe, A.; Decker, E.L.; Gorr, G.; Schween, G.; Reski, R. *Plant Cell Rep.*, **2002**, 20, 1135.
- Daniell, H. *Nat. Biotechnol.*, **1999**, 17, 855.
- Daniell, H.; Streatfield, S.J.; Wycoff, K. *Trend Plant Sci.*, **2001**, 6, 219.
- Gewolb, J. *Science*, **2002**, 295, 258.
- Staub, J.M.; Garcia, B.; Graves, J.; Hajdukiewicz, P.T.; Hunter, P.; Nehra, N.; Paradkar, V.; Schlittler, M.; Carroll, J.A.; Spatola, L.; Ward, D.; Ye, G.; Russell, D.A. *Nat. Biotechnol.*, **2000**, 18, 333.
- De Cosa, B.; Moar, W.; Lee, S.B.; Miller, M.; Daniell, H. *Nat. Biotechnol.*, **2001**, 19, 71.
- Thanavala, Y.; Yang, Y.F.; Lyons, P.; Mason, H.S.; Arntzen, C. *Proc. Natl. Acad. Sci. USA*, **1995**, 92, 3358.
- Arakawa, L.; Chong, D.K.X.; Langridge, W.H.R. *Nat. Biotechnol.*, **1998**, 16, 292.
- Okada, A.; Okada, T.; Ide, T.; Itoh, M.; Tanaka, K.; Takaiwa, F.; Toriyama, K. *Mol. Breed.*, **2003**, 12, 61.
- Molvig, L.; Tabe, L.M.; Eggum, B.O.; Moore, A.E.; Craig, S.; Spencer, D.; Higgins, T.J. *Proc. Natl. Acad. Sci. USA*, **1997**, 94, 8393.
- Smart, V.; Foster, P.S.; Rothenberg, M.E.; Higgins, T.J.V.; Hogan, S.P. *J. Immunol.*, **2003**, 171, 2116.
- Denson, J.; Kearney, C.M.; Hill, M.E.; Dawson, W.O. *Proc. Natl. Acad. Sci. USA*, **1991**, 88, 7204.
- Krebitz, M.; Wiedermann, U.; Essl, D.; Steinkellner, H.; Wagner, B.; Turpen, T.H.; Ebner, C.; Scheiner, O.; Breiteneder, H. *FASEB J.*, **2000**, 14, 1279.

- [47] Krebitz, M.; Wagner, B.; Ferreira, F.; Peterbauer, C.; Campillo, N.; Witty, M.; Kolarich, D.; Steinkellner, H.; Scheiner, O.; Breiteneder, H. *J. Mol. Biol.*, **2003**, *13*, 721.
- [48] Marillonnet, S.; Gritsch, A.; Gils, M.; Kandzia, R.; Klimyuk, V.; Gleba, Y. *Proc. Natl. Acad. Sci. USA*, **2004**, *101*, 6852.
- [49] Bohle, B.; Breitwieser, A.; Zwolfer, B.; Jahn-Schmid, B.; Sara, M.; Sleytr, U.B.; Ebner, C. *J. Immunol.*, **2004**, *172*, 6642.
- [50] Goldstein, D.A.; Thomas, J.A. *QJM*, **2004**, *97*, 705.
- [51] Breiteneder, H.; Krebitz, M.; Wiedermann, U.; Wagner, B.; Essl, D.; Steinkellner, H.; Turpen, T.H.; Ebner, C.; Buck, D.; Niggemann, B.; Scheiner, O. *Int. Arch. Allergy Immunol.*, **2001**, *124*, 48.
- [52] Holmberg, N.; Farres, J.; Bailey, J.E.; Kallio, P.T. *Gene*, **2001**, *5*, 115.
- [53] Huang, J.; Wu, L.; Yalda, D.; Adkins, Y.; Kelleher, S.L.; Crane, M.; Lonnerdal, B.; Rodriguez, R.L.; Huang, N. *Transgenic Res.*, **2002**, *11*, 229.
- [54] Scheller, J.; Guhrs, K. H.; Grosse, F.; Conrad, U. *Nat. Biotechnol.*, **2001**, *19*, 573.
- [55] Okuda, M.; Satoh, T.; Sakurai, N.; Shibuya, K.; Kaji, H.; Samejima, T. *J. Biochem.*, **1997**, *122*, 918.
- [56] Fiedler, U.; Phillips, J.; Artsaenko, O.; Conrad, U. *Immunotechnology*, **1997**, *3*, 205.
- [57] Menassa, R.; Kennette, W.; Nguyen, V.; Rymerson, R.; Jevnikar, A.; Brandle, J. *J. Biotechnol.*, **2004**, *108*, 179.
- [58] Maliga, P. *Curr. Opin. Plant Biol.*, **2002**, *5*, 164.
- [59] Maliga, P. *Annu. Rev. Plant Biol.*, **2004**, *55*, 289.
- [60] Hondred, D.; Walker, J.M.; Mathews, D.E.; Vierstra, R.D. *Plant Physiol.*, **1999**, *119*, 713.