

Among the problems of overproduction of recombinant proteins is the aggregation of the heterodimers and the misrefolding of the b2 domain, which is not only partially efficient, but also unstable, although bacteria are able to produce large amounts of recombinant antibodies in cell systems. This is the case for the production of the recombinant b2 domain, similar to the natural b2 domain of *Brasiliensis* latex. The production of antibodies from recombinant b2 domain isoenzymes (5). In the primary response to the antigen, research, rHev b 2 was used in experiments (6,7). Hev b 2 was significantly different in structures of allelic variants. The production of eight recombinant antibodies from patients. Pru av 2 was expressed in the

On the other hand, the loss of a strength for the protein. Allergens that lose their strength while retaining their structure are not suitable for such a strategy. The protein *Parietaria judaica* 1 (11). In addition, the protein is an allergen. Current research is focused on identifying epitopes on allergens that are not recognized by IgE-binding capacity. The loss of bonds after they are

Recombinant allergens are quickly becoming the reagents of choice for diagnosis and therapy of type I allergic diseases. Consequently, the different methods for the production of recombinant proteins that are available today are of great interest to allergologists. Without doubt, bacterial expression will continue to play a pivotal role. In addition, plant-based expression systems will be needed to overcome problems inherent in the *E. coli* systems and to allow the production of glycoallergens or allergens of more complex folding.

It is now generally accepted that the use of pure recombinant allergens in allergy diagnosis and treatment is able to overcome many of the difficulties inherent in natural extracts. The increase in prevalence of type I allergic diseases is making a greater demand on the quality of diagnostic and therapeutical products and on the number of recombinant allergens that need to be made available as their cDNAs become available. Recombinant allergens have been produced in different expression systems, *E. coli* being the most commonly used (1). A great variety of commercially available bacterial expression vectors exists. Bacterial systems are easy to handle, cost-effective and produce high yields of recombinant proteins. The most widely used technology to produce recombinant allergens is the gene fusion technology. This technology incorporates many features useful for easy cloning and purification of recombinant proteins.

Three of the most frequently used gene fusion affinity tags are glutathione S-transferase (GST), maltose binding protein (MBP), and the hexahistidyl tag. These commercial expression systems allow the rapid purification of fusion proteins by immobilized ligands. GST fusions bind strongly to glutathione-Sepharose, MBP fusions to an

amylose resin, and His<sub>6</sub> tagged proteins to immobilized metal ions such as Ni<sup>2+</sup>. Although these systems work well for a wide variety of proteins, problems in the expression may be encountered that make it necessary to switch to other systems. Sheffield and coworkers have replaced the incompatible polylinkers of pGEX4T1 (Pharmacia Biotech), pMAL-c2 (New England Biolabs) and pET15B (Novagen) with a uniform polylinker that allows simultaneous subcloning of genes into all three systems thereby creating a new family of *E. coli* gene fusion expression vectors of general interest to molecular allergologists (2). The polylinker that was introduced into the expression plasmids was derived from the baculo expression vector pFastBac-Hta (Life Technologies) (3). A very useful feature of this polylinker is the presence of a cleavage site for the tobacco etch virus (TEV) protease, a highly specific viral protease (4). The cleavage of the fusion protein is accomplished by the incubation with the recombinant TEV proteinase that itself contains a polyhistidine tract at its amino terminus. This allows the TEV proteinase to be separated from the cleaved product of interest.

Among the problems that can be encountered when using bacterial systems is the overproduction of recombinant proteins in the cytoplasm of *E. coli* leading to the aggregation of the heterologous proteins and to the formation of inclusion bodies. *In vitro* refolding of the biologically inactive recombinant protein is time-consuming and often only partially effective resulting in a low yield of the desired product. In addition, although bacterial expression systems represent the preferred tools for the production of recombinant allergens, there are allergens that are best expressed in eukaryotic systems. This is the case when plant-specific glycosylation or post-translational modifications of the recombinant allergen are needed to establish IgE-binding capability similar to the natural allergen. Hev b 2, a basic beta-1,3-glucanase and allergen of *Hevea brasiliensis* latex, comprises one non-glycosylated and two glycosylated isoenzymes. IgE antibodies from latex-allergic individuals predominantly recognized the glycosylated isoenzymes (5). In addition, the authors could show that the carbohydrate epitopes are primarily responsible for the IgE-binding determined by *in vitro* assays. In previous research, rHev b 2 produced in *E. coli* performed very poorly in RAST and ELISA experiments (6,7). The IgE-binding capability of natural and therefore glycosylated Hev b 2 was significantly higher than its recombinant *E. coli* counterpart (8,9). The structures of allergenic thaumatin-like proteins (TLPs) depend on the correct formation of eight conserved disulfide bonds as do their abilities to bind IgE from atopic patients. Pru av 2, an allergenic TLP from sweet cherry, lost its ability to bind IgE when expressed in the cytoplasm of *E. coli* (10).

On the other hand, such apparent weaknesses of bacterial systems can be turned into a strength for the production of hypoallergens for use as future therapeutic products. Allergens that lose their IgE-binding capacities when their correct molecular folding is lost while retaining their T-cell stimulatory capabilities represent a good example for such a strategy. The disruption of the disulfide bridges in Par j 1, a major allergen of *Parietaria judaica* pollen, resulted in the loss of most of its specific IgE-binding activity (11). In addition, no skin prick test reactivity was observed for the structurally altered allergen. Currently available data from crystallographic studies suggest that many IgE epitopes on allergens are conformational (12). It is therefore of interest to check the IgE-binding capacities of allergens whose structures are held together by disulfide bonds after they have been produced in the cytoplasm of *E. coli*. One such allergen of

interest would be Hev b 6.02, the hevein of the latex of *Hevea brasiliensis* (13). Likewise, allergens whose IgE-binding ability is dependent on the presence of certain carbohydrate moieties could be expressed in *E. coli* resulting in the loss of IgE epitopes, but not of the T-cell stimulatory abilities. One such candidate allergen is the above-mentioned Hev b 2.

Bacterial expression systems still remain the systems of choice to evaluate large quantities of constructs. This is the case when methods of genetic engineering are applied to produce new allergen molecules with reduced allergenicity but preserved immunogenicity. Such hypoallergens seem to quickly become the future therapeutic products for allergen-specific immunotherapy (14). DNA shuffling or molecular breeding is a new approach to generate allergen chimeras that are made up of DNA fragments from several homologous allergens but possess a reduced anaphylactogenic potential and an improved immunogenicity (15,16).

### Allergen expression in plants

Although bacterial expression systems are more robust than plant systems, they are not ideal for synthesizing eukaryotic proteins because of differences in metabolic pathways, protein processing, codon usage, and the formation of inclusion bodies (17). As plants are among the most economical producers of biomass, they provide an optimal system for the expression of recombinant proteins, even for the large scale production (18). In addition, they offer an eukaryotic protein modification machinery, allowing plant-specific glycosylation, folding, posttranslational modifications, and subcellular targeting (19). Especially for the expression of plant-derived allergens, these features support the production of recombinant allergens immunologically equivalent to their natural counterparts. Furthermore, proteins expressed by plants are free of human pathogens, such as bacterial endotoxins. Neither further testing of toxin concentrations nor further expensive purification steps for the elimination of the toxic contamination are needed to obtain safe vaccines for immunotherapy (19). Another benefit of the use of plant expression systems is the possibility to produce edible vaccines (20). Oral delivery eliminates injection-related hazards and is therefore easy to handle and to administer. Vaccines produced in edible parts of plants are easily grown in large scale, easily stored without chilling providing their own heat stable environment, and easily distributed, leading to the production of 'low-budget' vaccines (19). Several pharmaceutical products so far synthesized in plants are reviewed in Refs 18 and 21.

There are two different strategies for the production of recombinant proteins in plants: transient production using plant virus-based expression systems and stable insertion into the genome via *Agrobacterium tumefaciens*-mediated transformation or particle bombardment (21).

### Transient plant expression systems

The majority of plant viruses are single-stranded RNA viruses that encapsulate genomic RNA. The relatively small genomes are easy to manipulate *in vitro* and make it possible to engineer the viral genome as cDNA in bacterial plasmids.

An example for expression in plant is the expression of the tobacco etch virus (TEV) genome. The TEV genome (RdRp) translated protein (CP) both of which are required for the 17.5 kDa structural movement of the viral genome.

Another candidate is the type member of the tobacco etch virus (TEV) which encodes five open reading frames for cell-to-cell movement.

Foreign genes can be expressed in viral subgenomic proteins that need for the production of binding capacities have already been expressed in *E. coli* (11,12) such as the above-mentioned allergens.

Therefore, we used a virus-based expression system as Hev b 1 and Hev b 2 allergens. Furthermore, both belonging to the TLPs contain 16 amino acids for the overall fold.

Schematic diagram of the production of recombinant dual subgenomic proteins using the original subgenomic driving transcript and a hybrid vector (Fig. 1). The tobacco mild green virus (TMGV) through homologous recombination Scale Biology Corporation expression constructs of the original wild type.

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An example for a plant virus that has been extensively investigated for transient expression in plants is tobacco mosaic virus (TMV) (22), the type member of the tobamovirus group, a single-stranded, positive-sense RNA virus of about 6400 nucleotides. The viral genome codes for the RNA-dependent RNA polymerase (RdRp) translated right from the genomic RNA, a movement protein (MP) and a coat protein (CP) both translated from 3' coterminal subgenomic mRNAs (23). The 30 kDa MP is required for cell-to-cell movement of progeny viral RNA genomes. The CP, a 17.5 kDa structural protein, is produced at very high levels and is required for systemic movement of the virus through the vascular system in the plant and for encapsulation of the viral genome (23,24).

Another candidate for plant virus-derived expression vectors is potato virus X (25), the type member of the potexviruses. Its single plus-sense genomic RNA molecule encodes five open reading frames, coding for the RdRp, three proteins involved in cell-to-cell movement, and the CP (26).

Foreign genes may be expressed as read-through fusions with the viral CP or from viral subgenomic promoters, the method of choice for the production of recombinant proteins that need to be correctly folded, processed or glycosylated (27). This is useful for the production of biologically active recombinant plant-derived allergens with IgE-binding capacities identical to their natural counterparts. Although many allergens have already been successfully produced in expression systems other than plants, especially in *E. coli* (1), some of them are unable to bind patients' IgE when produced in *E. coli*, such as the above-mentioned rHev b 2.

Therefore, we started to focus on the production of allergens via transient virus-based expression vectors. We chose Bet v 1, the major birch pollen allergen (28), as well as Hev b 1 and Hev b 3, two *spina bifida*-associated latex allergens (29) as model allergens. Furthermore, the apple allergen Mal d 2 and the bell pepper allergen Cap a 1, both belonging to the group of thaumatin-like proteins (TLPs), were successfully produced via a plant virus-based expression vector in *Nicotiana benthamiana* plants (30,31). TLPs contain 16 conserved cysteine residues that form eight disulfide bonds essential for the overall folding as well as for their biological and allergenic activity (10).

Schematic diagrams of the TMV-based and the PVX-based vectors used for the production of recombinant allergens (28-31) are shown in Figure 1 and Figure 2. In the dual subgenomic plant virus expression vectors the foreign sequences are inserted after the original subgenomic CP promoter followed by a second, subgenomic CP promoter driving transcription of the CP gene itself. In the case of the TMV-derived 30B-based hybrid vector (Figure 1) (32), the second CP promoter is derived from the heterologous tobacco mild green mosaic virus (TMGMV) U5 to avoid loss of the foreign sequence through homologous recombination (33). This vector was kindly provided by Large Scale Biology Corporation (Vacaville, California). Interestingly, for PVX-derived expression constructs there is no need to introduce a heterologous CP promoter instead of the original wild-type one, as genome instability was seldom observed (25) (Figure 2).

There are two strategies for the infection of plants with recombinant plant virus vectors. In the case of the 30B-based vector, viral RNA transcripts from cDNA clones, *in*

*in vitro* transcribed under the control of the T7 promoter, serve as infectious templates for inoculation of plants (Figure 1) (27,34). In contrast, the PVX-based expression vector pgR106 (Figure 2), kindly provided by David Baulcombe (The Sainsbury Laboratory,

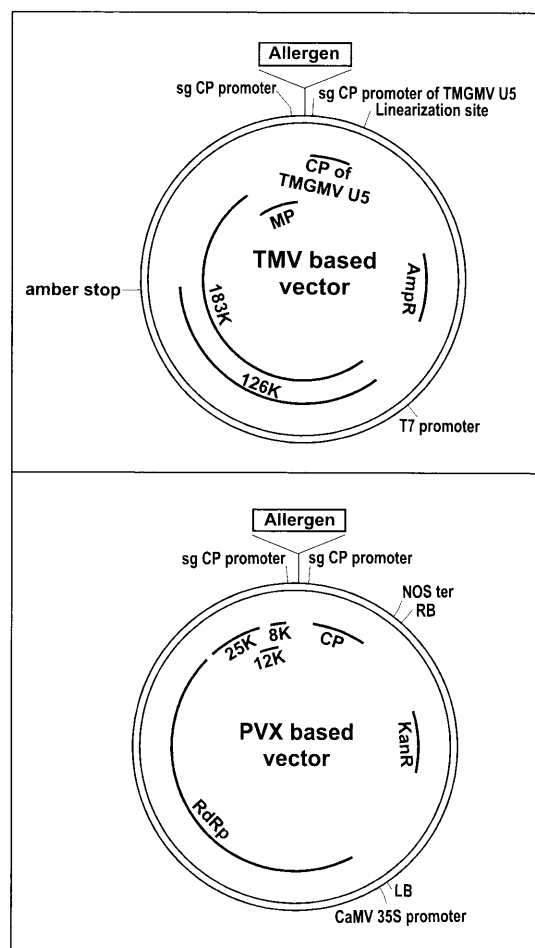


**Fig. 1.** Expression constructs of recombinant allergens introduced into a TMV-derived dual subgenomic vector (32). The open reading frames encoding several viral proteins, the 126K/183K RNA-dependent RNA polymerase, the movement protein (MP), and the heterologous tobacco mild green mosaic virus (TMGMV) U5-derived coat protein (CP) are indicated. The linearization site and the T7 promoter, both necessary for *in vitro* transcription to obtain infectious viral RNA, are shown. Subgenomic (sg) promoters controlling the transcription of the sequences coding for the CP and for the foreign allergen sequence (boxed) are also indicated.

**Fig. 2.** Expression construct of recombinant allergens in pgR106, a binary PVX-based expression vector (35,36). LB and RB, left and right border sequences of the T-DNA of *Agrobacterium tumefaciens*, surrounding the complete recombinant PVX genome including the allergen sequence of interest are indicated. The viral sequences coding for the RNA-dependent-RNA-polymerase (RdRp), the three movement proteins (25K, 12K, and 8K), the allergen of interest (boxed), and the viral coat protein (CP) are shown. Transcription is driven by the strong 35S promoter of cauliflower mosaic virus (CaMV) and terminated by the transcriptional terminator of the nopaline synthase gene of *A. tumefaciens* (NOS ter). Viral subgenomic (sg) promoters controlling the transcription of the allergen and the CP are also shown.

Norwich, UK), was designed to combine advantages of *Agrobacterium tumefaciens*-mediated transfection as well as of virus infection (35,36). The viral PVX-based cDNA is inserted into an agrobacterial plasmid, followed by transfection of plants by *A. tumefaciens* and integration of the recombinant PVX vector into the plant's genome. pgR106 derived from pGreen0000 (37) is a binary expression vector and provides replication origins for both *E. coli* and *A. tumefaciens*. The 25 bp imperfect repeats of agrobacterial origin, termed left border (LB) and right border (RB) that surround the cDNA of the PVX genome including the inserted foreign open reading frame, are necessary for the integration into the nuclear genome of the plant. Integration results in transcription of the PVX genome driven by the strong constitutive 35S-promoter derived from the cauliflower mosaic virus. These infectious recombinant PVX transcripts initiate the formation of virus particles, infecting the surrounding plant tissue (38,39).

The generation of transgenic plants is faster than the generation of transgenic animals. In addition, evaluations and safety studies are simpler and less expensive. Viruses have a wide host range and infect a wide range of plant species. The use of viral vectors for the insertion of the inserted allergen gene into the plant genome and the promoter (27).

So far, proteins of the size of the protein coat of plant viruses (40). Transgenic plant material containing a transient plant virus is biologically safe and does not spread mechanically and (27). Additionally, the wild type virus

Possible disadvantages levels due to decrease of the amounts of the homologous recombinants each inoculum production of the may occur, as virusinism although a genetic drift in fo

### Transgenic Plants

Stable transformation of plasmid DNA into the host cell is a prerequisite for DNA transfer or expression. Several methods have been used for this purpose. We investigated the immunogenicity of a B subunit (LTB) of *Yersinia enterocolitica* vaccine (45), both

The great advantage of the expression system - is that every positively selected gene of the coding sequence of the expression system can be used. Foreign genes amplified from genomic plants are being inserted into the resulting in uniform

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The generation of transiently virus-infected plants is faster and cheaper to establish than the generation of transgenic plants (27). It is therefore a useful method for rapid evaluations and screenings and to improve new expression constructs (36). As plant viruses have a wide host range, the same vector construct is compatible with different plant species. The method combines rapid amplification of the viral genome including the inserted allergen's sequence with high expression levels due to the strong viral CP promoter (27).

So far, proteins up to 80 kDa have been expressed. It is therefore unlikely, that the size of the protein will present a problem in transient expression using recombinant plant viruses (40). Even phytotoxic proteins that would hamper the development of the transgenic plant may be successfully produced by inoculating the mature plant via transient plant virus expression systems (41). Recombinant plant viruses are environmentally safe and do not represent a biohazard, as spreading of the virus occurs only mechanically and there is no efficient natural vector to transmit the virus in the field (27). Additionally, the foreign sequences will be eliminated by recombination to obtain the wild type virus (32,42).

Possible disadvantages of viral expression systems include (i) unpredictable expression levels due to gene silencing, a genetic control mechanism of plants that results in decrease of the expression level of the foreign sequence, or even in undetectable amounts of the recombinant allergen, and (ii) loss of the foreign sequence through homologous recombination yielding wild type virus. Therefore, in lab scale experiments each inoculated plant needs to be screened for viral infection as well as for the production of the foreign allergen. Furthermore, during multiple passages mutations may occur, as viral amplification is not subjected to eukaryotic proof reading mechanism although a study by Kearney and coworkers determined only a low level of genetic drift in foreign sequences during replication of viral RNA in plants (43).

### Transgenic Plants

Stable transformation of plants is characterized by the integration of the recombinant DNA into the genome of the plant either by *Agrobacterium tumefaciens*-mediated DNA transfer or by particle bombardment. Stable genetic transformation is the current method used for the production of edible vaccine (21). Tacket and coworkers investigated the immunogenicity in humans of recombinant bacterial heat labile enterotoxin B subunit (LTB) (44) as well as the human immune responses to a novel Norwalk virus vaccine (45), both of them delivered in transgenic potatoes.

The great advantage of transgenic plants – as compared to the transient plant expression system – is the stability of transformation, in parent as well as in the progeny plants. Every positively transformed plant produces the foreign protein, without possible loss of the coding sequence of interest as described for the transient plant virus-based expression systems (32,46). Furthermore, in contrast to what can be expected for foreign genes amplified by viral replication strategies, the inserted foreign genes in transgenic plants are not prone to mutations due to eukaryotic proof reading strategies resulting in uniform gene expression. Depending upon the promoters used, transgenic

proteins can be deposited throughout the plant in specific tissues or subcellular compartments, may be developmentally regulated or constitutively expressed (18). For example, Lauterslager and coworkers introduced a synthetic gene coding for LTB under the control of the tuber-specific patatin promoter into the potato genome resulting in the accumulation of the recombinant protein in potato tubers (47).

Konan and coworkers were the first who introduced the transgenic plant technology into the field of molecular biology of allergens (48). Ara h 2, a major peanut allergen, was integrated into the genome of peanut plants in order to downregulate the amount of Ara h 2 in the transgenic peanut plants due to transgene-induced gene silencing.

For the production of allergens in plants, gene silencing is a mechanism that could hamper high expression levels, but for the production of transgenic foods with less allergenic potential, this plant mechanism could be a future prospect.

Nevertheless, the production of transgenic plants is more time-consuming than the infection of plants with recombinant plant viruses. The generation of transient expression in plants takes only a few weeks from viral infection to harvest of the recombinant protein. In contrast, the establishment of transgenic plants from cell transformation to plant selection and plant growth lasts at least a few months. In addition, the view that transgenic plants represent environmental hazards is backed up by several studies (49,50).

Overcoming some of the drawbacks of transgenic plants, research has recently focused on the expression of recombinant proteins in the chloroplasts of the plant cell (51). The high copy number of the chloroplast genome leads to larger amounts of recombinant proteins produced in transgenic chloroplasts compared to expression levels in transgenic plants. Furthermore, as the chloroplast genome is maternally inherited, there is only minimal danger of spreading a transgene through pollen (51).

### Concluding remarks

Although most of the allergens known to date can be produced in bacterial expression systems, it is worthwhile to invest in the exploration of alternatives. Plant-based systems offer transient or stable expression of heterologous genes and are of high interest when plant-specific glycosylation or posttranslational modification of the recombinant allergen needs to be evaluated. Glycoallergens of plant origin can only be correctly produced as recombinant proteins in plants. They will be useful reagents to study the role of carbohydrate epitopes in type I allergy.

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## Discussion

**Vieths:** Did you h

**Breiteneder:** Yes, they are masked.

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## Discussion

**Vieths:** Did you have problems with His-tags in the proteins expressed in plants?

**Breiteneder:** Yes. Sometimes it happens that the His-tags are shortened, or sometimes they are masked.