

Plant virus expression systems for transient production of recombinant allergens in *Nicotiana benthamiana*

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Abstract

In recent years, several studies have demonstrated the use of autonomously replicating plant viruses as vehicles to express a variety of therapeutic molecules of pharmaceutical interest. Plant virus vectors for expression of heterologous proteins in plants represent an attractive biotechnological tool to complement the conventional production of recombinant proteins in bacterial, fungal, or mammalian cells. Virus vectors are advantageous when high levels of gene expression are desired within a short time, although the instability of the foreign genes in the viral genome may present problems. Similar levels of foreign protein production in transgenic plants often are unattainable, in some cases because of the toxicity of the foreign protein. Now virus-based vectors are for the first time investigated as a means of producing recombinant allergens in plants. Several plant virus vectors have been developed for the expression of foreign proteins. Here, we describe the utilization of tobacco mosaic virus- and potato virus X-based vectors for the transient expression of plant allergens in *Nicotiana benthamiana* plants. One approach involves the inoculation of tobacco plants with infectious RNA transcribed in vitro from a cDNA copy of the recombinant viral genome. Another approach utilizes the transfection of whole plants from wounds inoculated with *Agrobacterium tumefaciens* containing cDNA copies of recombinant plus-sense RNA viruses.

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1. Introduction

Plants are among the most economical producers of biomass. They only require sunlight, water, carbon dioxide, and minerals, but provide an optimal system for the expression of recombinant proteins free of contamination by bacterial toxins or animal pathogens. Furthermore, they offer a eukaryotic protein modification machinery, allowing subcellular targeting, proper folding, and post-translational modifications [1].

Foreign genes can be produced in plants by permanent insertion or by transient expression using virus-based vectors [2]. Viral replication rapidly amplifies genes that are engineered into a suitable position of the viral genome. Ideally, this results in high expression

levels of the recombinant protein throughout the whole plant [3]. Therefore, plant virus-based expression systems have been used for the inexpensive production of large quantities of correctly processed and functional therapeutic reagents such as biologically active and correctly glycosylated bovine follicle stimulating hormone [4], an experimental rabies vaccine having already been assessed in human volunteers [5], or the major inner capsid protein VP6 of rotavirus that is a cause of severe diarrhoea in children [6].

Foreign genes may be expressed as read-through fusions with a viral protein or from viral subgenomic promoters [7]. For read-through fusions, the sequence of interest is introduced immediately after an amber stop codon downstream of the viral coat protein (CP) coding sequence [7,8]. In the case of tobacco mosaic virus (TMV), its CP accumulates to as much as 10% of the dry weight of an infected leaf [9]. The modified CPs are then incorporated into virions displaying the foreign peptide

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on their surface. Such epitope presenting virions can even stimulate the immune system more effectively than a subunit vaccine [10]. For proteins that need to be post-translationally modified, dual subgenomic plant virus expression vectors are the system of choice. The foreign sequence is inserted after the subgenomic promoter originally driving transcription of the CP of the virus. This is followed by a second subgenomic promoter controlling transcription of the CP, the CP gene itself, and the 3' untranslated region of the viral genome.

The majority of plant viruses are single-stranded RNA viruses that encapsidate genomic RNA—per se directly infectious from inoculated leaf wounds. The relatively small genomes and the ease of manipulation of RNA viruses in vitro through reverse genetics make it possible to engineer the viral genome easily as DNA in bacterial plasmids. As plant viruses have a wide host range, the same vector construct is compatible for different plant species. Furthermore, few point mutations accumulate during multiple passages in the whole plant, although plant viruses do not use a proof-reading machinery for their replication [11].

In contrast to transgenic plants where transformation needs to be done only once, each generation of plants needs to be inoculated with the transient expression vector. This can be a great advantage for the production of phytotoxic proteins that would hamper the development of the transgenic plant. By inoculating the mature plant, problems of phytotoxicity can be circumvented as, for example, described by Kumagai et al. [12].

In this paper, we focus on the description of two plant virus expression systems, a TMV-based and a potato virus X (PVX)-based vector. In the case of the TMV-based vector, *Nicotiana benthamiana* plants are inoculated by in vitro RNA transcripts of the recombinant TMV genome. In contrast, the recombinant PVX vector is transferred to the plant's genome by *Agrobacterium tumefaciens* mediated inoculation.

Plant specific folding, processing, and glycosylation of heterologous proteins are important features provided by the plant expression system. Although many allergens have already been successfully produced in expression systems other than plants, especially in *Escherichia coli* [13], some allergens need a eukaryotic expression system for proper folding, disulfide-bond formation or glycosylation. These characteristics are necessary for the production of biologically active recombinant plant-derived allergens with IgE binding capacities identical to those of their natural counterparts. Problems with contaminating bacterial toxins of recombinant allergens produced in *E. coli* or hyperglycosylation of allergens produced in yeast are completely by-passed with plant expression systems. For the first time, we have introduced the transient plant virus expression system into the field of molecular biology of allergens. In these systems, the plants' eukaryotic ex-

pression machinery is combined with the ability of plus-sense single-stranded RNA viruses to rapidly amplify the foreign allergen's sequence and to promote the production of large amounts of the recombinant allergen of interest, in addition to proper folding and eukaryotic post-translational modifications. As examples, Bet v 1, the major birch pollen allergen [14], Hev b 1 and Hev b 3, two *Spina bifida* associated latex allergens [15], as well as the apple thaumatin like protein Mal d 2 [16] were successfully produced in our laboratory via a TMV-based expression vector in *N. benthamiana* plants.

2. The tobacco mosaic virus vector

TMV, the type member of the tobamovirus group, is a single-stranded, positive sense RNA virus of about 6400 nucleotides. The viral genome codes for two replicase-associated proteins translated right from the genomic RNA, and a movement protein (MP), and a CP translated from 3' coterminal subgenomic mRNAs. The genome of TMV employs two distinct strategies for protein expression: read-through of an amber stop codon and production of subgenomic RNAs [17]. The 5'-ORF of TMV is translated from the genomic RNA resulting in the accumulation of a 126 kDa protein. A translational read-through of an amber stop codon results in synthesis of a 183 kDa protein instead of the 126 kDa protein. These two proteins are first translated from the genomic RNA and comprise the viral RNA-dependent RNA polymerase (RdRp) synthesizing a full-length minus-sense copy of the genome. These copies are then used as a template for the amplification of plus-sense genomes and the subgenomic mRNAs for MP and CP synthesis [17]. The MP, a 30 kDa protein, is produced early in the infection cycle and is required for cell-to-cell movement of progeny viral RNA genomes by modifying the size exclusion limit of plasmodesmatal junctions [18,19]. Later in infection the production of genomic RNA, CP mRNA, and CP become the major events in the viral replication cycle. The CP, a 17.5 kDa structural protein produced at very high levels, is required for systemic movement of the virus through the vascular system in the plant and for encapsidation of the viral genome [20]. CP subunits spontaneously initiate encapsidation of the viral genomic RNA by binding to the origin of assembly, a specific RNA sequence that lies within the MP-ORF [17].

For the use of TMV as expression vector, viral RNA transcripts from cDNA clones serve as infectious templates for inoculation of plants [7,21]. Another method transfers viral cDNA into plant cells via agroinoculation, where cDNA of TMV is inserted into an agrobacterial plasmid and plants are transfected by *A. tumefaciens* [22]. As there is no efficient natural vector to transmit the virus in the field, recombinant TMV does

not represent a biohazard [7]. In addition, the foreign sequences will be eliminated by recombination which restores the wild type virus sequence [23,24].

Two general expression vector designs for TMV-based vectors were developed, CP fusion vectors and dual subgenomic expression vectors [7,25,26]. In CP fusion vectors, a “read-through sequence” is introduced immediately after the CP stop codon and before the ORF of interest [8,27]. Peptides of interest, e.g., the epitope of the murine hepatitis virus [28], are fused to the CP and are therefore incorporated into soluble virions presenting the peptide on the surface of the virion [7].

As fusion to the CP will not be desirable for some peptides or proteins, including those requiring post-translational maturation for active and/or stable protein formation, the 3′ portion of dual subgenomic vectors is designed with a heterologous tobamovirus CP subgenomic promoter, as well as the CP-ORF and the 3′ non-translated region of a divergent member of the tobamovirus group. The 5′ part of the vector contains the RdRP-ORF and the MP-ORF, as well as the subgenomic CP-promoter of TMV. The ORF of interest is inserted under the control of the original TMV CP-promoter. The frequency of homologous recombination, that could result in loss of the foreign sequence regenerating the original wild-type virus, is reduced by avoiding the duplication of TMV sequences through the design of hybrid vectors of divergent members of the tobamovirus group [7,23,25,26].

A schematic diagram of the TMV-based dual subgenomic vector described herein is shown in Fig. 1. This 30B-based hybrid vector is designed on the characteris-

tics identified as beneficial by Shivprasad et al. [23] in a study of a series of TMV expression vectors.

TMV-based vectors have been successfully applied for the production of medically relevant peptides or proteins in tobacco such as malaria epitopes [29], tumor-derived single chain variable fragments of antibodies [30,31], a vaccine candidate against hepatitis C virus [32], the extensively glycosylated bovine follicle stimulating hormone [4], or the neuropeptide nocistatin [33].

3. The potato virus X vector

Potato virus X (PVX), the type member of the potexviruses, is a filamentous rod-shaped virus, which contains a single plus-sense RNA molecule of about 6400 nucleotides. The RNA is capped at the 5′ end [34], polyadenylated at the 3′ end [35], and encodes five ORFs. Whereas ORF 1 is translated directly from the viral RNA, expression of the downstream cistrons takes place from a series of subgenomic RNAs, which are generated during PVX infection. The 5′ proximal cistron (ORF 1) encodes the RdRp (166 kDa). ORFs 2–4 consist of genes encoding a 25, a 12, and an 8 kDa protein, respectively, all involved in cell to cell movement [36]. These three cistrons, known as the “triple gene block,” are derived from overlapping ORFs. The 25 kDa protein is expressed from a functionally monocistronic mRNA, whereas the 12 and 8 kDa proteins are encoded in a bicistronic mRNA. The expression of the 8 kDa protein is achieved by leaky ribosome scanning through the 12 kDa ORF [37]. ORF 5 codes for the 25 kDa CP that is necessary for intercellular movement and efficient replication of PVX [38]. Before cell-to-cell transport, encapsidation of the viral RNA with CP is required, because only intact virions are transported through plasmodesmata during the infection and replication cycle of PVX [39,40].

Compared to many other plant virus vector systems, the PVX genome can accommodate large, heterologous sequence insertions, such as the β -glucuronidase (GUS) gene and the jellyfish green fluorescent protein [38,41]. In 1992, Chapman and co-workers [38] produced a plant virus vector from PVX containing two CP promoters, one of which directs the expression of foreign genes. As an example for the successful application of this vector, the *E. coli* GUS gene (1.9 kb) was expressed in *Nicotiana glauca* at similar or higher levels than had been achieved with transgenic plants [38]. Most of the viral RNA in the systemically infected tissue had retained the foreign gene insertion, genome instability of the PVX construct was seldom observed [38]. With some other viral vectors, homologous recombination during virus replication resulted in the generation of viral genomes that no longer incorporated the insert. With PVX this is not frequent enough to represent a significant problem

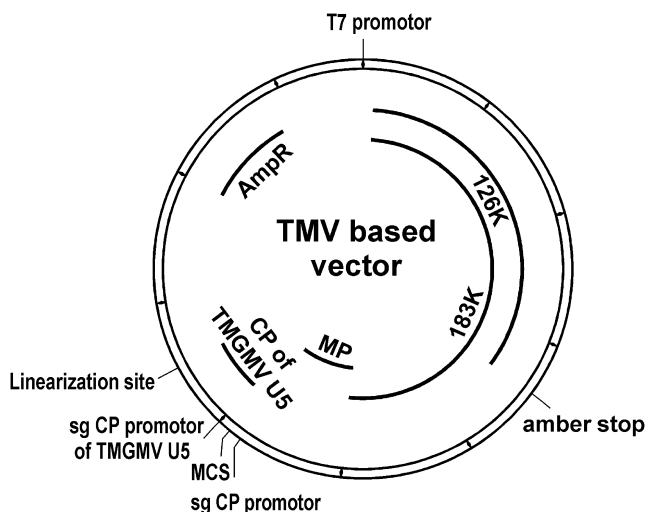


Fig. 1. Schematic diagram of a tobacco mosaic virus (TMV)-based dual subgenomic vector [23]. AmpR, ampicillin resistance; K, kilo-Dalton; 126K or 183K, RNA dependent RNA polymerase of TMV U1; MP, movement protein of TMV U1; sg, subgenomic; CP, coat protein of TMV U1; MCS, multiple cloning site; and TMGMV U5, tobacco mild green mosaic virus U5.

and there was no requirement to develop a chimeric vector, as was necessary with TMV [42].

The PVX-based expression vector pgR106 was designed to combine advantages of *A. tumefaciens* mediated transfection as well as of virus infection [43]. pgR106 is derived from pGreen0000 [44] and has a size of about 10 kb (Fig. 2) [43]. pgR106 is a binary expression vector and provides replication origins for both *E. coli* and *A. tumefaciens*. The ColEI origin of replication is responsible for the maintenance in *E. coli* and the broad host range replication origin pSa for *A. tumefaciens*. All pGreen derived vectors can only successfully replicate in *Agrobacterium* if another plasmid, pSoup, is co-resident in the same strain [44]. The relatively small pSa replication locus has been divided into a *trans*-acting pSa replicase gene, Rep A, and its target for replication, the replication origin (pSa ori). The Rep A gene is not present on pGreen and its derivatives, but on the compatible helper plasmid pSoup and provides replication functions in *trans* [44].

The PVX genome integrated into pgR106 contains a multiple cloning site that is surrounded by two sub-genomic RNA CP promoters between the triple gene block and the CP coding sequence (Fig. 2). The ORF of interest is inserted into the cloning site of the PVX within pgR106 in which its expression is controlled by one of the CP

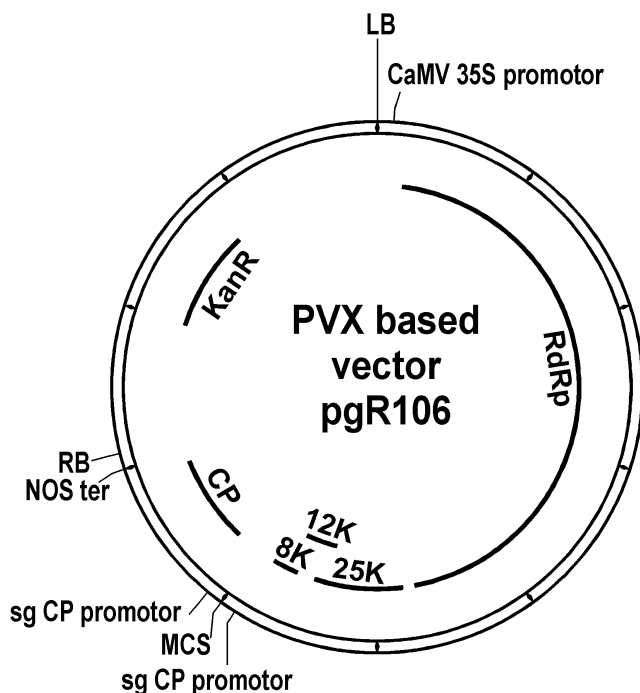


Fig. 2. Structure of pgR106, a binary potato virus X (PVX)-based expression vector [43]. KanR, kanamycin resistance; LB and RB, left and right border sequences of the T-DNA of *A. tumefaciens*; NOS ter, the transcriptional terminator of the nopaline synthase gene of *A. tumefaciens*; RdRp, viral RNA-dependent-RNA-polymerase; 25K, 12K, and 8K, viral movement proteins; CP, viral coat protein; CaMV, cauliflower mosaic virus; sg, subgenomic; and MCS, multiple cloning site.

promoters. The pgR106 plasmid also contains the strong constitutive 35S-promoter derived from the cauliflower mosaic virus that drives synthesis of infectious PVX transcripts in plants [3,45]. Furthermore, the vector harbors sequences of agrobacterial origin for the transfer of the expression construct to plant cells by *A. tumefaciens* and the integration into the nuclear genome of the plant. These are the 25 bp imperfect repeats termed left border (LB) and right border (RB) that surround the cDNA of the PVX genome with the inserted foreign ORF. Integration into the plant genome results in transcription of the PVX genome containing the sequence of interest. These transcripts initiate the formation of virus particles, infecting the surrounding plant tissue. The high reproduction levels of the virus result in high expression levels of the protein of interest [43].

4. Description of methods

A schematic presentation of the production of recombinant allergens with plant virus expression systems is given in Fig. 3.

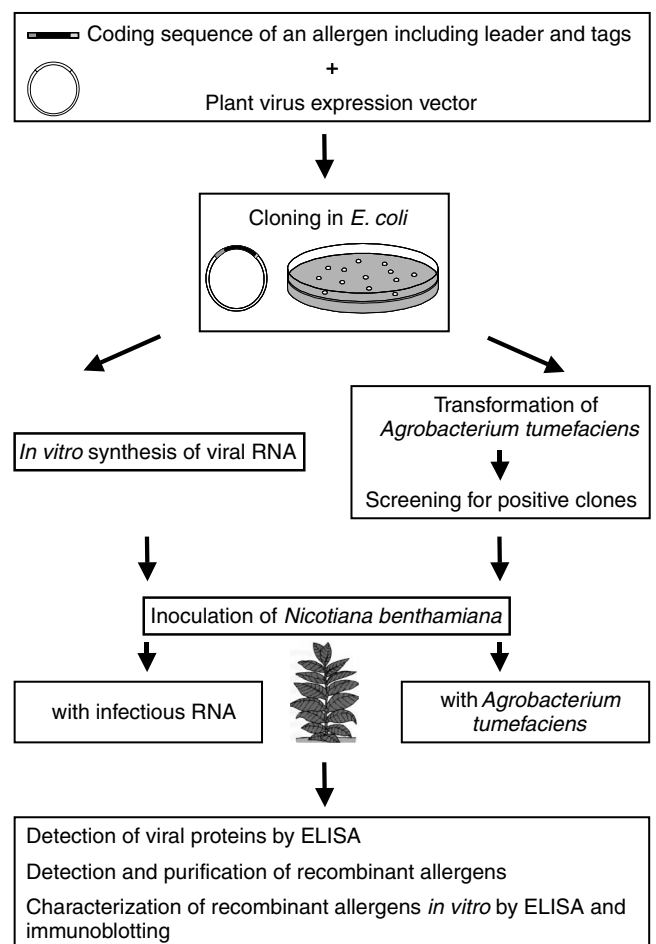


Fig. 3. Schematic presentation of the production of recombinant allergens with plant virus expression systems.

4.1. TMV

4.1.1. PCR amplification and cloning in *E. coli* of allergen encoding cDNAs

Genomic DNA preparations, RNA extracts, or plasmid preparations are used as templates for PCR amplification of allergen encoding sequences. Primers are designed to contain restriction sites corresponding to those included in the cloning sites of the vectors and a start codon when needed. If not provided in the allergen's sequence itself, nucleotides corresponding to the Kozak sequence need to be included in the primer design [46]. For shuttling the recombinant allergens into different subcellular compartments, sequences for subcellular location signals can also be introduced via primer design. For instance, the coding sequence for the SEKDEL retention signal was described to increase accumulation of the recombinant proteins in the endoplasmic reticulum [47–51]. Furthermore, affinity tags for easy purification or detection of the recombinant allergens in the infected plants, such as the hexahistidyl tag, can be introduced into the primer.

PCR amplification products are cloned into pGEM-T Easy Vector (Promega) or other PCR cloning vectors and sequenced. The DNA fragments containing the allergen's sequence are digested with restriction enzymes and ligated into the multiple cloning site of the TMV-based expression vector. XL-1 Blue *E. coli* cells transformed with the ligation product are then screened for the presence of TMV- and allergen sequences by PCR. Recombinant expression plasmids of positive clones are prepared using the alkaline lysis method [52].

4.1.2. In vitro production of infective recombinant TMV-RNA and inoculation of *N. benthamiana* plants

Template plasmids are linearized with a restriction enzyme followed by a digestion with Klenow fragment (Promega) to obtain blunt ends for in vitro transcription. Capped infectious viral RNA is generated in vitro from the linearized plasmid with the Ribomax Large Scale RNA Production System-T7 kit (Promega) and the Cap-Analog (Ambion) according to manufacturer's instructions. The integrity of the transcribed RNA is assessed by electrophoresis on a 1% agarose gel. After dusting *N. benthamiana* leaflets (2 per plant) lightly with carborundum powder, 2 µg in vitro transcripts are gently rubbed on their surface to initiate infection.

4.1.3. Detection of TMV infection by ELISA experiments

Plant tissue is ground with coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6, no pH adjustment required; 1 ml buffer per 100 mg tissue) in a mortar and pestle. Crude extracts are centrifuged for 6 min at 7000 rpm at room temperature. One hundred microliters plant extract per well, or positive and negative controls, respectively, are coated in duplicates on microtiter plates

overnight at 4 °C. After washing three times with 200 µl PBST (phosphate-buffered saline containing 0.05% Tween 20), 200 µl blocking buffer (PBST containing 0.5% non-fat dried milk powder) is added to each well. The plate is incubated at 37 °C for 1 h, washed as described above, and incubated with polyclonal anti-TMV antibody (#1065-05, Adgen, 100 µl/well, diluted 1:2000 in PBST containing 0.2% BSA) at 37 °C for 2 h. After washing three times, goat anti-rabbit IgG conjugated with alkaline phosphatase (#04007, Adgen, 100 µl per well, diluted 1:4000 in PBST/0.2% BSA) is added and the plate is incubated at 37 °C for 1 h. After washing four times with PBST, color development is performed using the Fast *p*-Nitrophenyl Phosphate Tablet Set (Sigma). The optical density is measured at 405 nm (550 nm as reference wavelength) by an ELISA reader (Dynatech MR 7000).

4.1.4. RNA isolation from TMV infected plants

Ten days after inoculation, systemic infection of plants and presence of allergen encoding RNAs are determined by RT-PCR. For RNA isolation, the TRI Reagent (MRC—Molecular Research Center) is used followed by chloroform extraction and iso-propanol precipitation. cDNA synthesis is performed with allergen-specific primers or vector-specific primers flanking the sequence of interest.

4.1.5. Protein extraction and detection of recombinant allergens

Between one and three weeks after inoculation, leaflets of infected plants are frozen in liquid nitrogen and pulverized. The powder is stirred in a modified version of the extraction buffer described by Arakawa et al. [53] [200 mM Tris, pH 8.0, 100 mM NaCl, 400 mM sucrose, 0.05% Tween 20, and 'Complete EDTA free protease inhibitor tablets' (Roche)] and centrifuged for 6 min at 7000 rpm and the supernatant loaded on SDS gels. SDS gels are Coomassie stained or transferred to nitrocellulose membranes for immunoblotting.

For large-scale production of recombinant proteins produced in virus infected *N. benthamiana* plants, the procedures described above can be easily scaled up. The complete plants are harvested, including leaf, stem, and root materials. Protein extraction is carried out with a threefold excess of the extraction buffer.

For purification of the recombinant allergens, conventional protein purification procedures such as ion exchange chromatography, gel filtration or affinity chromatography are used. For easy purification, affinity tags such as polyhistidyl tags (a nonahistidyl tag is more recommended than the common hexahistidyl tag due to its instability in some cases) or a FLAG tag can be introduced into the coding sequence of interest. For detection of the recombinant allergens produced in plants, immunoblotting or ELISA experiments are the methods of choice exploiting the binding abilities of the affinity

tags or the allergen itself. For example, polyhistidyl tagged proteins can be detected in immunoblots with an anti-pentahistidyl antibody. Briefly, nitrocellulose membranes are blocked for 45 min with TBST/1% fat-free milk powder and incubated with anti-Penta His (Qiagen, 1:2000 in TBST/milk) for 1 h at room temperature, followed by washing with TBST. After incubation with rabbit anti-mouse IgG + IgM conjugated with alkaline phosphatase (Jackson Immuno Research, 1:5000 in TBST/milk) for 1 h, the membranes are washed with TBST and treated with NBTC and BCIP for color development.

4.1.6. Immunological characterization of recombinant allergens in vitro

For a first immunological in vitro characterization of the recombinant allergens produced in virus infected plants, conventional procedures such as immunoblotting and ELISA experiments with patients' IgE are performed.

For IgE immunoblotting, plant extracts or the purified recombinant allergen are blotted onto nitrocellulose membranes and treated with blocking buffer (40 mM Na_2HPO_4 , 7 mM NaH_2PO_4 , 0.5% w/v fat-free milk powder, and 0.5% w/v Tween 20, pH 7.5). The membranes are then incubated with patients' sera or plasma (sera diluted in blocking buffer 1:6 or 1:8, plasma diluted 1:2) overnight at 4 °C. After washing with blocking buffer, the blots are incubated with ^{125}I -labelled anti-human IgE (IBL, Hamburg, Germany) overnight. Binding of patients' IgE to the allergens is visualized on Biomax MS film (Kodak, New York, USA). For inhibition studies, patients' sera are preincubated with the natural purified allergen overnight at 4 °C. The remainder of the procedure is identical to the immunoblot described above.

For IgE ELISA experiments, microtiter plates (Nunc Maxisorp) are coated overnight with plant extracts or the purified recombinant allergen in TBS. After washing with TBST (200 μl /well), plates are blocked with TBST/2% fat-free milk powder at room temperature for 2 h. Washing with TBST is followed by overnight incubation with patients' sera or patients' sera preincubated with different amounts of the purified allergen. After washing with TBST, alkaline phosphatase conjugated anti-human IgE (BD Pharmingen, 1:1000 diluted in TBST) is applied to the microtiter plates and incubated for 2 h at room temperature. Color development is performed using the Fast *p*-Nitrophenyl Phosphate Tablet Set (Sigma). The optical density is measured at 405 nm (550 nm as reference wavelength) using an ELISA reader (Dynatech MR 7000).

4.2. PVX

Cloning strategies in *E. coli* including PCR amplification and primer design are described in the chapter on TMV related methods above.

4.2.1. Transformation of *A. tumefaciens*

For our studies, we use the *A. tumefaciens* strain UIA 143, a derivative of the original progenitor wild-type strain C58 deficient in DNA repair and recombination through a mutation in the *recA* gene [54]. Strain UIA 143 has been transformed with the disarmed Ti plasmid pMP90. Other *A. tumefaciens* strains that support the use of pGreen or its related plasmid pgR106 with the help of pSoup are LBA4404, GV2260, GV3280, AGL-1, and EHA105 [44]. The PVX-based vector pgR106, that contains the inserted allergen encoding DNA sequence, and the helper plasmid pSOUP are introduced simultaneously into *Agrobacterium* UIA 143 cells by mixed electroporation (200 Ω , 1.5 V, and 25 μF , Gene Pulser II System, Bio-Rad Laboratories). After recovery at 28 °C in SOC-medium for 2 h, cells are plated on LB-medium supplemented with 50 $\mu\text{g}/\text{ml}$ kanamycin (a resistance that is located on pgR106) and 5 $\mu\text{g}/\text{ml}$ tetracycline (a resistance provided by pSOUP). Colony growth usually requires incubation on plates for four days at 28 °C. Positive colonies, identified by PCR screening with vector- as well as allergen-specific primers, are then grown overnight in 500 ml LB-medium.

4.2.2. Transfection of *N. benthamiana* plants

About four-week-old *N. benthamiana* plants approximately at a 10-leaf stage are suitable hosts for transfection with agrobacteria to subsequently produce viral RNA. Veins of two leaves per plant are wounded carefully by pricking with a needle. Stem and leaves of the plant are then submerged for twenty minutes into the overnight culture of a positive *Agrobacterium* clone containing the pgR106/allergen construct and the two helper plasmids. The 500 ml overnight culture can be used for more than one plant. The *N. benthamiana* plants treated in that way are then placed in the dark in a humid atmosphere for 24 h to recover from the treatment. Thereafter, plants are kept in a growth chamber under 16 h of daylight at 22 °C.

4.2.3. Detection of PVX infection by ELISA experiments

Approximately 10 days after the inoculation of the plants, the PVX-CP can be detected by a triple antibody sandwich ELISA (Adgen). According to the protocol, the polyclonal anti-PVX coating antibody (#1048-05, Adgen) is diluted 1:1000 in coating buffer (15 mM Na_2CO_3 , 35 mM NaHCO_3 , pH 9.6, no pH adjustment required) and 100 μl is added per well and incubated at 37 °C for 4 h. In the meantime, plant extracts are prepared. Per 100 mg of tissue, 1 ml general extraction buffer (2% polyvinylpyrrolidone, 0.2% BSA, 10 mM Na_2SO_3 , 3 mM NaN_3 , 0.05% Tween 20, 137 mM NaCl, 1.5 mM KH_2PO_4 , 8 mM Na_2HPO_4 , and 3 mM KCl, no pH adjustment necessary) is used for grinding in a

mortar and pestle. Crude extracts are centrifuged for 6 min at 7000 rpm at room temperature. After washing three times with 200 µl PBST (phosphate-buffered saline containing 0.05% Tween 20), 100 µl plant extract and a positive and negative control are added in duplicates to each coated well. The microtiter plate is incubated at 4 °C overnight and washed again as described above. One hundred microliters monoclonal anti-PVX probe antibody (#1048-05, Adgen) diluted 1:1000 in PBST containing 0.2% BSA is added and incubated at 37 °C for 2 h. After washing again, rabbit anti-rat IgG antibody conjugated with alkaline phosphatase (#04001, Adgen) is diluted 1:4000 in PBST/BSA, added to the wells (100 µl each), and incubated at 37 °C for 1 h. Now the plate is washed four times to ensure that all unbound conjugate is removed. Color development is performed using the Fast *p*-Nitrophenyl Phosphate Tablet Set (Sigma) and the optical density is measured at 405 nm (550 nm as reference wavelength) using an ELISA reader (Dynatech MR 7000).

The description of the protocols for RNA isolation of infected plants, for protein extraction as well as for the detection and characterization of recombinant allergens can be found in the chapter on TMV related methods above.

5. Concluding remarks

Overall, this report aims to demonstrate that plant virus vectors have the potential for becoming a useful tool to express recombinant allergens in plants, especially when plant-specific folding and glycosylation of the recombinant proteins are of importance. Not only are transfected leaves one of the most economical sources of biomass, but making therapeutic proteins in bacterial, fungal or cultured mammalian cells requires more costly incubation techniques and sterile conditions. In addition, time-consuming procedures needed to produce stably transformed plants can be avoided by using plant virus vectors. Although it will probably not work for all proteins, contained lab-scale production and industrial-scale production of some important allergens are definitely possible. We are still in the empirical stage of plant allergen expression by viral vectors. Whether such a strategy will lead to a more frequent application in the field of molecular allergology remains to be seen. Further studies are needed to thoroughly evaluate transient plant expression systems as tools for the production of recombinant plant allergens. In summary, plant virus vectors offer an alternative to express foreign proteins. In the more distant future, even edible vaccines for type I allergy may be developed to modulate the immune system to induce tolerance to allergens upon oral delivery.

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References

- [1] C. Cunningham, J.R. Porter, *Recombinant Proteins from Plants: Production and Isolation of Clinically Useful Compounds*, Humana Press, Totowa, New Jersey, 1998.
- [2] A.M. Walmsley, C.J. Arntzen, *Curr. Opin. Biotechnol.* 11 (2000) 126–129.
- [3] H.B. Scholthof, K.B.G. Scholthof, A.O. Jackson, *Annu. Rev. Phytopathol.* 34 (1996) 299–323.
- [4] D. Dirnberger, H. Steinkellner, L. Abdennebi, J.J. Remy, D. van de Wiel, *Eur. J. Biochem.* 268 (2001) 4570–4579.
- [5] V. Yusibov, D. Hooper, S. Spitsin, N. Fleysh, R. Kean, T. Mikheeva, D. Deka, A. Karasev, S. Cox, J. Randall, et al., *Vaccine* 20 (2002) 3155.
- [6] G.J. O'Brien, C.J. Bryant, C. Voogd, H.B. Greenberg, R.C. Gardner, A.R. Bellamy, *Virology* 270 (2000) 444–453.
- [7] G.P. Pogue, J.A. Lindbo, W.O. Dawson, T.H. Turpen, in: S.B. Gelvin, R.A. Schilperoort (Eds.), *Plant Molecular Biology Manual*, Kluwer Academic Publishers, Dordrecht, The Netherlands, 1998, pp. 1–38.
- [8] H. Hamamoto, Y. Sugiyama, N. Nakagawa, E. Hashida, Y. Matsunaga, S. Takemoto, Y. Watanabe, Y. Okada, *Biotechnology (N Y)* 11 (1993) 930–932.
- [9] R.J. Copeman, J.R. Hartman, J.C. Watterson, *Phytopathology* 59 (1969) 1012–1013.
- [10] G.P. Lomonosoff, W.D.O. Hamilton, *Curr. Top. Microbiol. Immunol.* 240 (1999) 177–190.
- [11] C.M. Kearney, J. Donson, G.E. Jones, W.O. Dawson, *Virology* 192 (1993) 11–17.
- [12] M.H. Kumagai, J. Donson, G. Della-Cioppa, D. Harvey, K. Hanley, L.K. Grill, *Proc. Natl. Acad. Sci. USA* 92 (1995) 1679–1683.
- [13] R. Hiller, S. Laffer, C. Harwanegg, M. Huber, W.M. Schmidt, A. Twardosz, B. Barletta, W.M. Becker, K. Blaser, H. Breiteneder, et al., *FASEB J.* 16 (2002) 414–416.
- [14] M. Krebitz, U. Wiedermann, D. Essl, H. Steinkellner, B. Wagner, T.H. Turpen, C. Ebner, O. Scheiner, H. Breiteneder, *FASEB J.* 14 (2000) 1279–1288.
- [15] H. Breiteneder, M. Krebitz, U. Wiedermann, B. Wagner, D. Essl, H. Steinkellner, T.H. Turpen, C. Ebner, D. Buck, B. Niggemann, et al., *Int. Arch. Allergy Immunol.* 124 (2001) 48–50.
- [16] M. Krebitz, B. Wagner, F. Ferreira, C. Peterbauer, N. Campillo, M. Witty, D. Kolarich, H. Steinkellner, O. Scheiner, H. Breiteneder, *J. Mol. Biol.* 329 (2003) 721–730.
- [17] W.O. Dawson, K.M. Lehto, *Adv. Virus Res.* 38 (1990) 307–342.
- [18] C.M. Deom, K.R. Schubert, S. Wolf, C.A. Holt, W.J. Lucas, R.N. Beachy, *Proc. Natl. Acad. Sci. USA* 87 (1990) 3284–3288.
- [19] C.M. Deom, M. Lapidot, R.N. Beachy, *Cell* 69 (1992) 221–224.
- [20] W.O. Dawson, P. Bublick, G.L. Grantham, *Phytopathology* 78 (1988) 783–789.

- [21] W.O. Dawson, D.L. Beck, D.A. Knorr, G.L. Grantham, *Proc. Natl. Acad. Sci. USA* 83 (1986) 1832–1836.
- [22] T.H. Turpen, A.M. Turpen, N. Weinzettl, M.H. Kumagai, W.O. Dawson, *J. Virol. Methods* 42 (1993) 227–239.
- [23] S. Shivprasad, G.P. Pogue, D.J. Lewandowski, J. Hidalgo, J. Donson, L.K. Grill, W.O. Dawson, *Virology* 255 (1999) 312–323.
- [24] S. Rabindran, W.O. Dawson, *Virology* 284 (2001) 182–189.
- [25] T.H. Turpen, S.J. Reinf, in: C. Cunningham, J.R. Porter (Eds.), *Recombinant Proteins from Plants: Production and Isolation of Clinically Useful Compounds*, vol. 3, Humana Press, Totowa, New Jersey, 1998, pp. 89–102.
- [26] V. Yusibov, S. Shivprasad, T.H. Turpen, W. Dawson, H. Koprowski, *Curr. Top. Microbiol. Immunol.* 240 (1999) 81–94.
- [27] J.M. Skuzeski, L.M. Nichols, R.F. Gesteland, J.F. Atkins, *J. Mol. Biol.* 218 (1991) 365–373.
- [28] M. Koo, M. Bendahmane, G.A. Lettieri, A.D. Paoletti, T.E. Lane, J.H. Fitchen, M.J. Buchmeier, R.N. Beachy, *Proc. Natl. Acad. Sci. USA* 96 (1999) 7774–7779.
- [29] T.H. Turpen, S.J. Reinf, Y. Charoenvit, S.L. Hoffman, V. Fallarme, L.K. Grill, *Biotechnology (N Y)* 13 (1995) 53–57.
- [30] A.A. McCormick, M.H. Kumagai, K. Hanley, T.H. Turpen, I. Hakim, L.K. Grill, D. Tuse, S. Levy, R. Levy, *Proc. Natl. Acad. Sci. USA* 96 (1999) 703–708.
- [31] C. Vaquero, M. Sack, J. Chandler, J. Drossard, F. Schuster, M. Monecke, S. Schillberg, R. Fischer, *Proc. Natl. Acad. Sci. USA* 96 (1999) 11128–11133.
- [32] L.G. Nemchinov, T.J. Liang, M.M. Rifaat, H.M. Mazyad, A. Hadidi, J.M. Keith, *Arch. Virol.* 145 (2000) 2557–2573.
- [33] A.A. Lim, S. Tachibana, Y. Watanabe, S.M. Wong, *Gene* 289 (2002) 69–79.
- [34] N. Sonenberg, A.J. Shatkin, R.P. Ricciardi, M. Rubin, R.M. Goodman, *Nucleic Acids Res.* 5 (1978) 2501–2512.
- [35] S.Y. Morozov, V.G. Gorbulev, V.K. Novikov, A.A. Agranovskii, Y.V. Kozlov, J.G. Atabekov, A.A. Baev, *Dokl. Akad. Nauk SSSR* 259 (1981) 723–725.
- [36] M.J. Huisman, H.J. Linthorst, J.F. Bol, J.C. Cornelissen, *J. Gen. Virol.* 69 (1988) 1789–1798.
- [37] J. Verchot, S.M. Angell, D.C. Baulcombe, *J. Virol.* 72 (1998) 8316–8320.
- [38] S. Chapman, T. Kavanagh, D. Baulcombe, *Plant J.* 2 (1992) 549–557.
- [39] S. Santa Cruz, A.G. Roberts, D.A. Prior, S. Chapman, K.J. Oparka, *Plant Cell* 10 (1998) 495–510.
- [40] O. Fedorkin, A. Solov'yev, N. Yelina, A. Zamyatnin Jr., R. Zinovkin, K. Makinen, J. Schiemann, S. Yu Morozov, *J. Gen. Virol.* 82 (2001) 449–458.
- [41] D.C. Baulcombe, S. Chapman, S. Santa Cruz, *Plant J.* 7 (1995) 1045–1053.
- [42] J. Donson, C.M. Kearney, M.E. Hilf, W.O. Dawson, *Proc. Natl. Acad. Sci. USA* 88 (1991) 7204–7208.
- [43] L. Jones, A.J. Hamilton, O. Voinnet, C.L. Thomas, A.J. Maule, D.C. Baulcombe, *Plant Cell* 11 (1999) 2291–2301.
- [44] R.P. Hellens, E.A. Edwards, N.R. Leyland, S. Bean, P.M. Mullineaux, *Plant Mol. Biol.* 42 (2000) 819–832.
- [45] C. Porta, G.P. Lomonosoff, *Mol. Biotechnol.* 5 (1996) 209–221.
- [46] M. Kozak, *Nucleic Acids Res.* 9 (1981) 5233–5262.
- [47] S. Munro, H.R. Pelham, *Cell* 48 (1987) 899–907.
- [48] T.A. Haq, H.S. Mason, J.D. Clements, C.J. Arntzen, *Science* 268 (1995) 714–716.
- [49] A. Schouten, J. Roosien, F.A. van Engelen, G.A. de Jong, A.W. Borst-Vrens, J.F. Zilverentant, D. Bosch, W.J. Stiekema, F.J. Gommers, A. Schots, et al., *Plant Mol. Biol.* 30 (1996) 781–793.
- [50] A. Schouten, J. Roosien, J.M. de Boer, A. Wilmink, M.N. Rosso, D. Bosch, W.J. Stiekema, F.J. Gommers, J. Bakker, A. Schots, *FEBS Lett.* 415 (1997) 235–241.
- [51] C.I. Wandelt, M.R. Khan, S. Craig, H.E. Schroeder, D. Spencer, T.J. Higgins, *Plant J.* 2 (1992) 181–192.
- [52] J. Sambrook, E.F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, 1989.
- [53] T. Arakawa, D.K. Chong, J.L. Merritt, W.H. Langridge, *Transgenic Res.* 6 (1997) 403–413.
- [54] S.K. Farrand, S.P. O'Morchoe, J. McCutchan, *J. Bacteriol.* 171 (1989) 5314–5321.