

# Rapid Production of Recombinant Allergens in *Nicotiana benthamiana* and Their Impact on Diagnosis and Therapy

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## Key Words

Allergens, recombinant · Tobacco mosaic virus · *Nicotiana benthamiana* · Bet v 1 · Hev b 1 · Hev b 3

## Abstract

**Background:** Type I allergies are immunological disorders that afflict a quarter of the world's population. Recombinant allergens have improved the diagnosis of allergic diseases and allow the formulation of new therapeutic approaches. Over 50% of all allergens are of plant origin. **Objective:** We have applied a novel method of overexpressing plant allergens in the tobacco-related species *Nicotiana benthamiana*. **Method:** This method is based on the use of a chimeric tobacco mosaic virus that harbors a foreign gene sequence and directs its transcription after the infection of the host plant. **Results:** We have expressed the model allergen Bet v 1, the major birch pollen allergen, and two *Hevea brasiliensis* latex allergens, the spina-bifida-associated allergens Hev b 1 and Hev b 3, in *N. benthamiana* using such a viral vector. Bet v 1, Hev b 1 and Hev b 3 produced by this method were recognized by patients' IgE suggesting that the plant-produced allergens were properly folded. Nonpurified Bet v 1 expressed in *N. benthamiana* leaves had

the same immunogenicity as purified Bet v 1 expressed in *Escherichia coli* or natural Bet v 1 when tested in a murine model of type I allergy. **Conclusion:** We conclude that this plant expression system offers a viable alternative to fermentation-based production of allergens in bacteria or yeasts.

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## Introduction

Plants are widely used as biomanufacturing systems to produce proteins of pharmaceutical value. In contrast to the conventional production of transgenic plants, plant viral vectors offer a rapid production system for generating substantial amounts of heterologous proteins by transient expression. An attractive candidate virus is the tobacco mosaic virus (TMV), a well-characterized single-stranded RNA virus that replicates extrachromosomally and redirects protein synthesis of host cells to express high levels of a protein of interest throughout the plant [1]. TMV-based vectors have been successfully applied for the production of medically relevant peptides or proteins in tobacco such as tumor-derived single chain variable fragments of antibodies [2].

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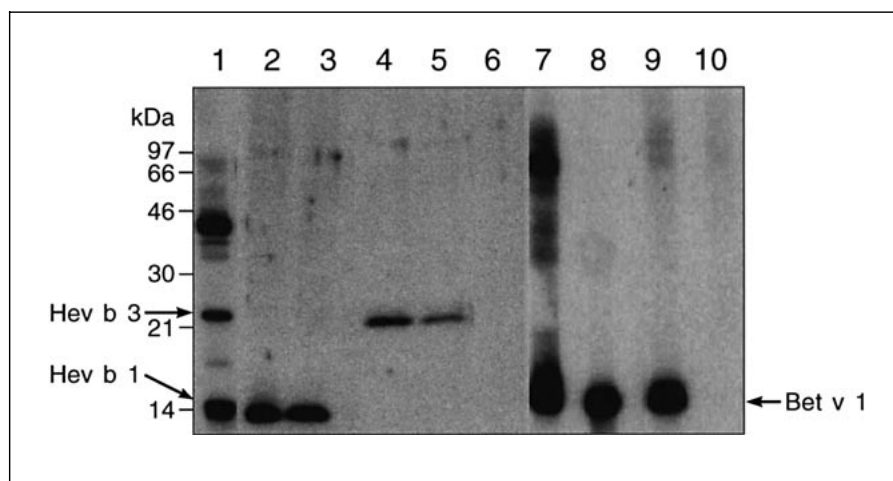
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**Fig. 1.** IgE immunoblot detection of Hev b 1 and 3 in a *H. brasiliensis* latex extract (lane 1), and in extracts of *N. benthamiana* leaves infected with recombinant TMV (Hev b 1: lanes 2, 3; Hev b 3: lanes 4, 5), detection of Bet v 1 in a birch pollen extract (lane 7) and in extracts of *N. benthamiana* leaves infected with recombinant TMV (lanes 8, 9). A serum pool of latex-allergic individuals was used to detect Hev b 1 and Hev b 3, a serum of birch pollen-allergic individuals for Bet v 1. Controls shown are of the pool of the latex allergics (lane 6) or of the pool of the birch pollen allergics (lane 10) on extracts of plants infected with nonrecombinant TMV.



## Materials and Methods

### *In vitro* Production of Infective Recombinant Viral RNA and Inoculation of *Nicotiana benthamiana* plants

The open reading frame (ORF) of Bet v 1 (acc. No. X15877) was amplified by PCR introducing a *SalI* restriction site at the start and a *PstI* site at the stop codon of the ORF. The ORFs of Hev b 1 (acc. No. X56535) and Hev b 3 (acc. No. AJ223388) were amplified by PCR introducing a *PacI* restriction site at the start and an *AvrII* site at the stop codons. The digested Bet v 1 fragment was ligated into the plasmid p4GD-PL [1], the digested Hev b 1 and 3 fragments into the recently developed TMV expression vector pBSG735A66 (provided by Large Scale Biology Corporation, Vacaville, Calif.). pBSG735A66 is based on characteristics identified as beneficial by Shivprasad et al. [3] in a study of a series of TMV expression vectors. Capped infectious viral RNA was generated *in vitro* from purified plasmid DNAs by using a T7 RNA polymerase kit (Promega, Madison, Wisc.). Infection of *N. benthamiana* plants was initiated by manually damaging leaves and applying approximately 2 µg of the *in vitro* transcribed RNAs.

### Protein Extraction and Immunoblot Studies

Two weeks after inoculation, systemic infection of plants and presence of allergen-coding RNAs were determined by RT-PCR on total RNA extracted from newly formed upper leaves. Leaves from plants with positive results were frozen in liquid nitrogen, ground to a fine powder, and extracted with a 10 mM potassium phosphate buffer at pH 8.0 containing 2 mM EDTA, 10 mM diethyldithiocarbamate, 2% (w/v) polyvinylpyrrolidone and 1 mM PMSF. The supernatants obtained from centrifuging the extract at 40,000 *g* for 1 h were dialyzed against 10 mM potassium phosphate buffer, pH 8.0 and stored at –20 °C. Pollen-derived natural Bet v 1 was isolated from *Betula verrucosa* pollen [4]. Serum samples from 5 birch pollen-allergic patients or from 5 spina bifida patients with latex allergy were pooled and used to check leaf extracts for the presence of the recombinant allergens. Atopic patients were characterized by a clinical history of allergic symptoms to birch pollen or latex products, by positive skin prick test responses to birch pollen or latex extract and by positive immunoblot results.

### Cutaneous Type I Hypersensitivity Reaction in BALB/c Mice

BALB/c mice were injected intraperitoneally with purified rBet v 1 produced in *Escherichia coli*, rBet v 1 in a crude *N. benthamiana* leaf extract, or nBet v 1 in a crude birch pollen extract. All antigens were adsorbed to Al(OH)<sub>3</sub>. Control mice were injected with crude leaf extract from plants infected with the viral vector without the Bet v 1 coding sequence. Immunizations were performed at days 0, 14 and 28. Seven days after the last immunization, intradermal skin tests were performed with birch pollen extract and *E. coli* rBet v 1 as previously described [5]. After 20 min, the mice were sacrificed, and the color intensity of the reaction was compared with the positive control.

## Results

### Expression of Bet v 1, Hev b 1 and Hev b 3 in *N. benthamiana* plants

Inoculation of *N. benthamiana* plants with *in vitro* transcripts derived from p4GD-Bet v 1, pBSG735A66-Hev b 1 or pBSG735A66-Hev b 3 resulted in symptomatic infection after 7 days. Direct sequencing of the RT-PCR products confirmed the correct sequence of the allergens' mRNAs. Twenty-one days after inoculation, leaf material was harvested and extracted. Ten microliters of the supernatants from infected leaves were separated by SDS-PAGE and analyzed by immunoblotting using pooled sera from birch pollen- or latex-allergic patients. The respective serum pools displayed IgE binding to Hev b 1 and 3 present in a *Hevea brasiliensis* latex extract (fig. 1, lane 1) or in *N. benthamiana* leaves (fig. 1, lanes 2–5), to natural Bet v 1 present in birch pollen (fig. 1, lane 7) or to Bet v 1 in *N. benthamiana* (fig. 1, lanes 8 and 9). No IgE binding could be detected to leaf protein extracts

from control plants using the latex- or birch pollen-allergic serum pool (fig. 1, lanes 6 and 10, respectively).

#### *Cutaneous Type I Hypersensitivity Reaction in BALB/c Mice*

BALB/c mice immunized with purified rBet v 1 produced in *E. coli*, crude *N. benthamiana* leaf extract containing rBet v 1 or crude BP extract displayed equally strong immediate-type skin reactions 20 min after intradermal injection of rBet v 1 or BP extract. No allergen-specific skin reactions were elicited in the control mice immunized with crude leaf extract from *N. benthamiana* plants infected with the nonrecombinant viral vector.

#### Discussion

There is a growing need for the experimental, diagnostic and clinical use of pure allergens. Expression of plant allergens in a homologous system poses less problems concerning correct folding, glycosylation and solubility of recombinant proteins. It was our aim to establish a plant-based expression system for the production of plant-derived allergens as an alternative to *E. coli* or yeast-based systems. The birch pollen allergen Bet v 1 was chosen as a model allergen to evaluate transient expression in plants infected by recombinant viral vectors. To further assess the potential of this system, two *Hevea* latex allergens, Hev b 1 and Hev b 3, were transiently expressed in *N. ben-*

*thamiana*. The binding of IgE from sera from birch pollen- and latex-allergic patients to the allergens indicated the equivalence of IgE epitopes between the natural and the recombinant allergens. We have previously established a mouse model for type I allergy to birch pollen [5] that is also a useful tool to evaluate new systems for recombinant allergen production. We have applied this model to compare immune responses after immunization with standard purified rBet v 1 produced in *E. coli* to Bet v 1 produced in the described plant system. Notably, the application of Bet v 1 in a total plant protein extract, i.e. unpurified, induced immune responses that were equal in terms of skin test reactivity to those following immunization with purified rBet v 1.

The application of TMV-based vectors for the expression of plant allergens will have a major impact on laboratory research, therapeutic treatments and possibly commercial biotechnology where otherwise hard to produce allergens are concerned. For the production of such proteins, plants will provide an optimal system at reduced costs and potential large-scale production compared to other fermentation-based technologies.

#### Acknowledgment

The financial support of the Austrian Science Fund (grant No. P12838-GEN) is gratefully acknowledged.

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