

Rapid production of the major birch pollen allergen Bet v 1 in *Nicotiana benthamiana* plants and its immunological *in vitro* and *in vivo* characterization

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ABSTRACT Type I allergies are immunological disorders that afflict a quarter of the world's population. Improved diagnosis of allergic diseases and the formulation of new therapeutic approaches are based on the use of recombinant allergens. We describe here for the first time the application of a rapid plant-based expression system for a plant-derived allergen and its immunological characterization. We expressed our model allergen Bet v 1, the major birch pollen allergen, in the tobacco-related species *Nicotiana benthamiana* using a tobacco mosaic virus vector. Two weeks postinoculation, plants infected with recombinant viral RNA containing the Bet v 1 coding sequence accumulated the allergen to levels of 200 µg/g leaf material. Total nonpurified protein extracts from plants were used for immunological characterizations. IgE immunoblots and ELISA (enzyme-linked immunoassay) inhibition assays showed comparable IgE binding properties for tobacco recombinant (r) Bet v 1 and natural (n) Bet v 1, suggesting that the B cell epitopes were preserved when the allergen was expressed in *N. benthamiana* plants. Using a murine model of type I allergy, mice immunized with crude leaf extracts containing Bet v 1 with purified rBet v 1 produced in *E. coli* or with birch pollen extract generated comparable allergen-specific IgE and IgG1 antibody responses and positive type I skin test reactions. These results demonstrate that nonpurified Bet v 1 overexpressed in *N. benthamiana* has the same immunogenicity as purified Bet v 1 produced in *E. coli* or nBet v 1. We therefore conclude that this plant expression system offers a viable alternative to fermentation-based production of allergens in bacteria or yeasts. In addition, there may be a broad utility of this system for the development of new and low-cost vaccination strategies against allergy.—Krebitz, M., Wiedermann, U., Essl, D., Steinkellner, H., Wagner, B., Turpen, T. H., Ebner, C., Scheiner, O., Breiteneder, H. Rapid production of the major birch pollen allergen Bet v 1 in *Nicotiana benthamiana*

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POLLEN FROM TREES of the order *Fagales*—in particular, birch—are a major source of allergenic proteins that cause immunoglobulin E (IgE)-mediated diseases in early springtime (1). IgE-mediated type I allergy is a serious and increasing health problem in the western world. Consequently, there is a rising need for accurate diagnosis applying purified, well-defined, and standardized allergenic molecules. Recombinant DNA techniques are applied to produce such proteins with immunological characteristics identical to the naturally occurring proteins (2–4). Recombinant allergens significantly improve accuracy and reliability of diagnosis of type I allergies (2–4). Bet v 1, the major allergen of birch pollen, was the first plant allergen that was cloned, sequenced (5), and expressed in *Escherichia coli* (6, 7). Bet v 1 was subsequently studied in great detail regarding its molecular biology (8, 9) and immunological *in vitro* (10–12) and *in vivo* characteristics (13–15). In addition, the 3-dimensional structure of Bet v 1 has been determined (16). In industrialized countries 5 to 7% of the population suffer from type I allergies to tree pollen (17). More than 95% of tree pollinosis patients produce IgE directed against Bet v 1. Therefore, Bet v 1 represents a model allergen for the study of type I diseases and is the allergen of choice to evaluate new expression systems.

So far, Bet v 1 has been expressed in *E. coli*, as no post-translational modifications of the natural allergen could be detected by mass spectrometry (18).

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The yield using the *E. coli* expression system pMW175/BL21(DE3) was ~10 mg of purified recombinant Bet v 1 per liter of bacterial culture (7). To obtain pure *E. coli* recombinant (r) Bet v 1 for *in vitro* use, several purification steps were necessary including ion-exchange and hydrophobic interaction chromatography as *E. coli* extracts contain considerable amounts of lipopolysaccharides (7). Although bacterial expression systems have the advantage of speed and abundant production, they are limited in their ability to express properly folded soluble proteins without the need of denaturation and renaturation.

In comparison to *E. coli*, yeast-based expression systems offer the advantages of a eukaryotic folding machinery, the absence of endotoxin, and higher yields of the recombinant protein. Expression in the yeasts *Pichia pastoris* and *Saccharomyces cerevisiae* were successfully applied for allergens from avocado (Pers a 1, ref 19), latex (Hev b 7, ref 20), olive pollen (Ole e 1, ref 21), cockroach (Bla g 4, ref 22), house dust mite (Der p 2, ref 23), and bermuda grass pollen (Cyn d 1, ref 24) resulting in yields of up to 50 mg per liter of culture. However, several researchers have experienced difficulties expressing certain plant proteins in *E. coli* or yeast, resulting in low or no yield or the inability of the expressed allergen to bind IgE. These problems are caused by the lack of a plant-specific protein folding machinery in bacteria and yeasts and by differences in glycosylation and/or codon usage. To address these points and to circumvent low yields or the toxicity of *E. coli* extracts and the need for complex purification procedures, plant allergens are ideally expressed in a plant-based system.

In particular, expression of heterologous proteins in plants to produce edible vaccines has generated great interest in recent years. The hepatitis B surface antigen (25), a Norwalk virus coat protein (26), the *E. coli* heat-labile enterotoxin B subunit (27), and the cholera-toxin B subunit (28) were stably produced in their native immunogenic forms either in tobacco or potato plants transformed by the *Agrobacterium* method. A disadvantage of this type of plant expression was the low expression level of the recombinant heterologous proteins. In contrast, plant viral vectors offer a rapid production system for generating substantial amounts of heterologous proteins without having to go through the process of establishing transgenic plants.

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 heterologous proteins throughout the plant (29). TMV-based vectors have been successfully applied to produce medically relevant peptides or

proteins in tobacco such as malaria epitopes (30) or tumor-derived single chain variable fragments of antibodies (31).

Here we present for the first time the TMV-directed expression of an allergen in the tobacco-related species *Nicotiana benthamiana* and characterization of the immune response against this plant-produced allergen in humans *in vitro* and in mice *in vivo*. Furthermore, we demonstrate that Bet v 1 present in crude extracts of *N. benthamiana* plants displays identical immunological parameters to those of purified rBet v 1 produced in *E. coli*.

MATERIALS AND METHODS

Construction of the vector p4GD-Bet v 1

Polymerase chain reaction (PCR) primers specific for the Bet v 1 coding sequence (EMBL accession no. X15877, ref 7) were designed with a *SalI* restriction site at the 5'-end and a *PstI* restriction site at the 3'-end in order to allow subsequent cloning (sense strand primer: 5'-TAGTCCGACATGGGTGTTTCACCTTACGA-3'; antisense strand primer: 5'-AGACTGCAGGTTGTAGGCATCGGAGTGTGC-3' (priming regions underlined, *SalI* and *PstI* sites in italics). PCR was performed in a 100 µl volume containing 0.5 µg purified Bet v 1 DNA prepared from clone pMW175/Bet v 1 (7), 25 pmol each of sense and antisense primer, 2.0 mM MgCl₂, 0.2 mM of each dNTP (dATP, TTP, dCTP, and dGTP), and 2.5 U AmpliTaq DNA Polymerase in PCR buffer II (Perkin Elmer Cetus, Norwalk, Conn.). PCR amplification was carried out on a Perkin Elmer thermal cycler (GenAmpPCR System 2400). The amplification procedure included a denaturation step at 94°C for 3 min, 30 cycles of 45 s strand separation at 94°C, 30 s annealing at 63°C for 30 s, and 30 s extension at 72°C, followed by an elongation step of 10 min at 72°C. The PCR product was purified by agarose gel electrophoresis, digested with *SalI* and *PstI*, and ligated into respective sites of the p4GD-PL vector containing a hybrid fusion of TMV-U1 and tobacco mild green mosaic virus (29). The ligated DNA was used for the transformation of competent *E. coli* XL1-Blue cells (Clontech, Palo Alto, Calif.).

In vitro transcription of capped recombinant viral RNA

Plasmid p4GD-Bet v 1 was prepared for *in vitro* transcription by linearization with *SfiI*. Linearized plasmid DNA was purified over spin columns (QIAquick Spin, Qiagen, Calif.) and made blunt-ended by the DNA polymerase I Klenow Fragment (New England Biolabs Inc., Boston, Mass.) for 15 min. Capped infectious RNA was generated from 1 µg plasmid template using T7 RNA polymerase (Promega Corporation, Madison, Wis.). Transcription reactions contained 40 mM HEPES-KOH (pH 8.0), 24 mM MgCl₂, 2 mM spermidine, 10 mM dithiothreitol, 0.5 mM each of ATP, CTP, and UTP, 0.2 mM GTP, 0.5 mM cap analog G(5')ppp(5')G (New England Biolabs), 1 U/µl RNasin (Promega), 1 µg linearized DNA, and 0.6 U/µl T7 RNA polymerase in a 50 µl volume. Reactions were incubated for 2 h at 37°C. Synthesis of RNA was analyzed by gel electrophoresis.

Inoculation of *N. benthamiana* plants

Carborundum-dusted leaves of *N. benthamiana* were manually inoculated with 10 µl of the transcription reaction containing

2 µg of *in vitro* transcribed viral RNA. Immediately after inoculation, plants were rinsed with water and placed in growth chambers. Plants were grown under 16 h of daylight at 22°C and observed daily for signs of infection such as necrotic local lesions or systemic vein yellowing and variable leaf mottling. These experiments were performed following the recommendations given by the European Commission for GLP regarding genetically modified organisms.

Reverse transcriptase (RT)-PCR analysis of transfected plants

Two weeks after inoculation, total RNA was isolated by grinding newly formed upper leaves with visual viral symptoms in liquid nitrogen to powder and extracting with phenol/chloroform (TRI-Reagent, Molecular Research Center, Cincinnati, Ohio). Reverse transcription was performed on 1 µg total RNA using the GeneAmp RNA PCR kit (Perkin Elmer Cetus). The cDNA synthesis was primed with a TMV-specific antisense strand primer downstream the insertion site (5'-TTTTTCCTTTTGTTCG-3'). For RT-PCR, the same primer and a primer upstream of the insertion site (5'-GATGATGATTCGGAGGCTACT-3') were used. For control experiments, total RNA from uninfected *N. benthamiana* and from plants infected with viral RNA that did not contain the Bet v 1 coding sequence was subjected to RT-PCR. Sequence analysis of the RT-PCR product was performed using the Thermosequenase Fluorescent Labeled Primer Cycle Sequencing kit (Amersham Life Science Ltd, London, U.K.) and the LI-COR DNA sequencer model 4000L (LICOR, Lincoln, Nebraska).

Protein extractions

Leaves, stems, and roots of infected *N. benthamiana* plants were harvested 21 days postinoculation. For Western blot analysis, plant materials were frozen in liquid nitrogen and ground to a fine powder in a Waring Blendor. The powder was then stirred overnight at 4°C in a 10-fold excess (w/v) of 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 by centrifugation at 40,000 g and 4°C for 1 h were filtered, dialyzed against 10 mM potassium phosphate buffer, pH 8.0, and stored at -20°C until use. Ten grams of birch pollen (Allergon AB, Vålinge, Sweden) were extracted in the same way, except that the extract was dialyzed against phosphate-buffered saline (PBS), pH 7.0. Pollen-derived natural (n) Bet v 1 was purified by affinity chromatography with an immobilized anti-Bet v 1 monoclonal antibody, followed by reversed-phase high-performance liquid chromatography (6).

Sera from atopic and nonatopic individuals and monoclonal antibodies

Serum or plasma samples from five individual birch pollen-allergic patients, a standard serum pool of eight birch pollen-allergic patients, and a pool of 10 nonatopic volunteer donors were used in this study. Atopy was determined by a clinical history of allergic symptoms to birch pollen, by positive skin prick test responses to birch pollen extract, and by positive radioallergosorbent test (RAST) (classes 3.5–5.0) to birch pollen. Nonatopic subjects were healthy individuals without any allergic history, negative RAST results, and negative skin prick test responses to birch pollen allergens. A murine monoclonal anti-Bet v 1 antibody (BIP1, ref 17) was raised against natural Bet v 1. Hybridoma supernatant was stored at -20°C and used in a 1:10 dilution of TBS [50 mM Tris/150

mM NaCl/0.5% Tween 20/0.5% bovine serum albumin (BSA); pH 7.4] for immunoblots.

Protein quantification

Protein concentrations were determined by the Bradford method according to the Bio-Rad-Microassay Procedure (Bio-Rad, Hercules, Calif.). A standard curve was produced with known concentrations of BSA (Sigma-Aldrich, St. Louis, Mo.). Ultraviolet absorption at 280 nm was used to estimate sample protein concentration. Protein quantification of tobacco rBet v 1 was performed by densitometric analysis using image analysis software (E.A.S.Y., Enhanced Analysis System, Herolab, Wiesloch, Germany) to calculate band intensities and comparing to a standard dilution series of *E. coli* rBet v 1.

Immunoblot studies

Protein extracts from birch pollen, purified rBet v 1 produced in *E. coli* (7), extracts from *N. benthamiana* plants expressing Bet v 1, and extracts from control plants infected with the viral vector alone were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 12% polyacrylamide gels and transferred to nitrocellulose membranes by electroblotting according to standard protocols (32). Protein extracts were adjusted to contain 1 µg Bet v 1 per lane. Blots were then incubated with sera (diluted 1:5 in blocking buffer), plasma (diluted 1:2) from birch pollen-allergic patients, or BIP1, a mouse monoclonal anti-Bet v 1 IgG antibody (17). Bound IgE was detected by ¹²⁵I-labeled rabbit anti-human IgE (RAST RIA, Pharmacia, Uppsala, Sweden), diluted 1:10, and visualized on Biomax MS film (Kodak, Rochester, N.Y.). Alternatively, to detect BIP1, alkaline phosphatase-conjugated rabbit anti-mouse IgG (Jackson Immuno Lab. Inc., West Grove, Pa.), diluted 1:1000, was used and developed with the BCIP (5-bromo-4-chloro-3-indolyl phosphate)/NBTC (4-nitroblue tetrazolium chloride) reagent solutions (Boehringer Mannheim, Mannheim, Germany).

ELISA and ELISA cross-inhibition experiments

Microtiter plates (Nunc-Immuno Plate, Nalge Nunc International, Roskilde, Denmark) were coated with 5 µg/ml of the monoclonal antibody BIP1 (17) as catching antibody in PBS. After blocking with PBS, 0.05% Tween 20, 1% BSA, plates were incubated with increasing concentrations of purified nBet v 1, *E. coli* rBet v 1 or rBet v 1 from crude *N. benthamiana* leaf extract ranging from 0.125 µg/ml to 2.0 µg/ml. Crude leaf extract from *N. benthamiana* infected with the viral vector not harboring the Bet v 1 coding sequence was applied as control in the same serial dilution as Bet v 1 containing *N. benthamiana* leaf extract. After washing five times with PBS and 0.05% Tween 20, rabbit anti-Bet v 1 IgG (polyclonal serum, diluted 1:10,000 in PBS/0.05% Tween 20/0.5% BSA) was applied to the microtiter plates and incubated for 3 h at room temperature. Washing was followed by addition of peroxidase-conjugated mouse anti-rabbit IgG (Jackson Immuno Lab. Inc., 1:2000 PBS 0.05% Tween 20/0.5% BSA). Color development was performed using 0.1% ABTS (azinoethylbenzothiozoline sulfonic acid substrate, Sigma-Aldrich) and the optical density was measured at 405 nm (490 nm as reference wavelength) after 30 min using an ELISA reader (Dynatech MR 7000, Alexandria, Va.). Adequate controls were performed for each incubation step by replacing the respective ligand by PBS (data not shown).

For cross-inhibition experiments, the Bet v 1-specific serum pool was diluted 1:10 in Tris-buffered saline (TBS), 0.05% Tween 20, 1% BSA, and preincubated (overnight at 4°C) with

40.0, 20.0, 10.0, 5.0, 2.5, 1.25, and 0 $\mu\text{g}/\text{ml}$ of purified nBet v 1, protein extract from *N. benthamiana* plants expressing Bet v 1, or BSA. Microtiter plates were coated with 5.0 $\mu\text{g}/\text{ml}$ BIP1, followed by 2 $\mu\text{g}/\text{ml}$ of purified nBet v 1 or rBet v 1 in a crude *N. benthamiana* leaf extract. Afterward, preincubated sera were applied onto the BIP1/nBet v 1 and BIP1/tobacco rBet v 1-coated plates and incubated overnight at 4°C. After washing, the plates were incubated with alkaline phosphatase-conjugated mouse anti-human IgE antibody (PharMingen, San Diego, Calif.) and developed with the substrate *p*-nitrophenyl phosphate. The color reaction was measured as described above, except that the reference wavelength was at 550 nm. Controls with TBS and a normal human serum pool assured the specification of the IgE detection system (data not shown).

Sensitization of BALB/c mice

Seven-week-old female BALB/c mice were purchased from Charles River (Sulzfeld, Germany). Mice ($n=5/\text{group}$) were either injected with 1 μg purified rBet v 1 produced in *E. coli*, crude *N. benthamiana* leaf extract containing 1 μg Bet v 1, or crude birch pollen extract containing 1 μg Bet v 1. The antigens adsorbed to $\text{Al}(\text{OH})_3$ were intraperitoneally administered in 150 μl volumes. Control mice were injected with 150 μl crude leaf extract from *N. benthamiana* infected with the viral vector not harboring the Bet v 1 coding sequence. Immunizations were performed three times, on days 0, 14, and 28. Six days after the last immunizations blood samples were taken from the tail vein, serum prepared and stored at -20°C until analyzed.

Cutaneous type I hypersensitivity reaction in BALB/c mice

Seven days after the last immunization (i.e., on day 35 of the immunization protocol), intradermal skin tests were performed as described previously (14). One hundred microliters of 0.5% Evans blue (Sigma-Aldrich) were injected intravenously (i.v.) into the tail vein of the mice. Subsequently, 30 μl of birch pollen extract (2.5 $\mu\text{g}/\text{ml}$) and *E. coli* rBet v 1 (2.5 $\mu\text{g}/\text{ml}$) were injected intradermally into the shaved abdominal skin. The mast cell degranulation compound 48/80 (20 $\mu\text{g}/\text{ml}$, Sigma) was used as a positive control. PBS was injected as a negative control. After 20 min, the mice were killed and the color intensity of the reaction was compared with the individual positive control on the inverted skin.

Determination of allergen-specific antibody levels in sera of BALB/c mice by ELISA

Allergen-specific antibody levels in mouse sera were determined by ELISA (14). In brief, microtiter plates were coated with *E. coli* rBet v 1 (2 $\mu\text{g}/\text{ml}$). Serum samples diluted 1/1000 for IgG1, 1/500 for IgG2a, and 1/10 for IgE antibodies were applied in 100 μl volumes. After washing, anti-mouse IgG1, IgG2a, and IgE antibodies (1 $\mu\text{g}/\text{ml}$, PharMingen) were applied overnight at 4°C. Detection of bound antibodies was performed with peroxidase-conjugated mouse anti-rat IgG antibodies (1/1000, Jackson Immuno Lab. Inc.). ABTS (Sigma-Aldrich) was used for color development and the absorbance was measured at 405 nm with a Dynatech microplate reader. The antibody levels were expressed in optical density units. The baseline levels derived from the preimmune sera were individually subtracted from the respective immune sera. Statistical analyses were performed with the Mann Whitney *U* test.

Determination of allergen-specific IgE antibody levels by passive cutaneous anaphylaxis (PCA)

Passive cutaneous anaphylactic reactions were performed in Wistar rats. Pooled sera from *E. coli* rBet v 1 or tobacco rBet v 1 immunized BALB/c mice were diluted 1:4 and a 1:20 in PBS and intradermally injected in a 50 μl volume on both sides of the back of the rats. Preimmune sera, in a 1:4 dilution, were injected as negative controls. After 24 h, 1 mg of birch pollen extract in 1 ml 0.5% Evans blue was i.v. injected into each animal. The mast cell degranulation compound 48/80 was intradermally injected as a positive control (50 μl of 1 mg/ml). The reaction was read 25 min after the antigen challenge. The diameter of the reaction was measured and the area and the color intensity was evaluated on the inside of the skin.

RESULTS

Construction of p4GD-Bet v 1 and *in vitro* transcription

In this study we have constructed a plant viral vector that directs the expression of the allergen Bet v 1 in *N. benthamiana* plants. The coding sequence of Bet v 1 was placed under the control of the TMV-U1 coat protein subgenomic promoter. The insertion of the Bet v 1 sequence was confirmed by DNA sequence analysis. A recombinant clone containing the correct Bet v 1 coding sequence was named p4GD-Bet v 1. Infectious viral RNA was produced by *in vitro* transcription with T7 RNA polymerase. By running an aliquot of the transcription reaction on a denaturing gel, the transcript length was estimated to be 7.1 kb (data not shown), which is in good agreement with the expected size of the TMV genome (6651 bp) harboring the Bet v 1 coding sequence (483 bp).

Expression of Bet v 1 in *N. benthamiana* plants

Mechanical inoculation of *N. benthamiana* plants with *in vitro* transcripts derived from p4GD-Bet v 1 DNA resulted in systemic infection 7 days postinoculation visible as mild leaf deformations, some variable leaf mottling, and growth retardation. RT-PCR was performed with RNA isolated from newly formed upper leaves 2 wk postinoculation. Direct sequencing of the RT-PCR product confirmed the correct sequence of the Bet v 1 mRNA transcribed from the viral subgenomic promoter in systemically infected leaves. Twenty-one days postinoculation, leaf, stem, and root material was harvested, weighed, and extracted. Ten microliters of the supernatant from infected leaves were separated by SDS-PAGE and stained by Coomassie brilliant blue. As Bet v 1 and the abundant viral coat protein migrated at approximately the same molecular mass range (17.5 kDa), a differentiation between the two proteins was not possible.

Subsequently, expression of tobacco rBet v 1 was

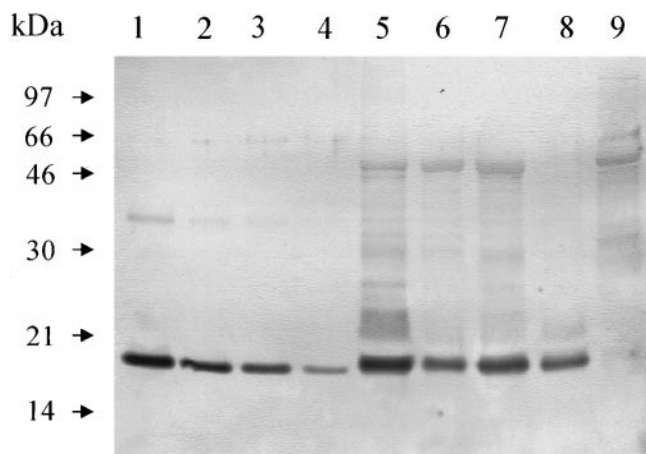


Figure 1. Binding of BIP1, a mouse monoclonal anti-Bet v 1 IgG antibody, to a dilution series of purified rBet v 1 produced in *E. coli* and to rBet v 1 transiently expressed in different *N. benthamiana* tissues. Immunoblotting shows BIP1 binding to 0.25 µg (lane 1), 0.1 µg (lane 2), 0.05 µg (lane 3), and 0.01 µg (lane 4) purified rBet v 1 from *E. coli* and to rBet v 1 present in extracts of upper leaves (lane 5), lower leaves (lane 6), stems (lane 7), and roots (lane 8) of *N. benthamiana* plants infected with the viral vector harboring the Bet v 1 coding sequence. No BIP1 binding to proteins in extracts of *N. benthamiana* leaves infected with the viral vector without the Bet v 1 coding sequence could be detected (lane 9).

analyzed by Western blotting using the anti-Bet v 1 monoclonal antibody BIP1 (**Fig. 1**). Plant tissue was homogenized in a 10-fold excess of buffer (w/v). Ten microliters of the supernatant resulting from the centrifugation were loaded per lane corresponding to ~1 mg fresh tissue. A dilution series of *E. coli* rBet v 1 (0.25 µg, 0.1 µg, 0.05 µg, 0.01 µg) was loaded on the same gel to estimate the amounts of rBet v 1 expressed in the different tissues. BIP1 detected tobacco rBet v 1 in protein extracts of upper leaves (**Fig. 1**, lane 5), lower leaves (the sites of infection; **Fig. 1**, lane 6), stems (**Fig. 1**, lane 7), and roots (**Fig. 1**, lane 8) 21 days postinoculation. In contrast, no BIP1 binding could be observed to proteins in extracts from *N. benthamiana* plants in-

fectured with viral RNA without the Bet v 1 insertion (**Fig. 1**, lane 9). Tobacco rBet v 1 amounts present in the different plant tissues were visualized by the intensity of the BIP1 binding and calculated by densitometric comparison to the BIP1 binding intensity to the *E. coli* rBet v 1 dilution series (**Fig. 1**, lanes 1–4). Tobacco rBet v 1 present in 10 µl supernatant extracted from the various tissues was calculated at 250 ng in upper leaves, in lower leaves at 50 ng, in stems at 200 ng, and in roots at 75 ng. Consequently, Bet v 1 was calculated to be present at 250 µg/g fresh upper leaves (0.025%). Total soluble protein extracted from leaves was determined by the method according to Bradford to be 10 mg/g fresh leaf. Therefore, rBet v 1 was accumulated to 2.525% of the soluble protein in leaves.

Immunoblots experiments

Immunoblot analyses of birch pollen extract (**Fig. 2**, lanes 1), purified *E. coli* rBet v 1 (**Fig. 2**, lanes 2), protein extract of *N. benthamiana* leaves expressing Bet v 1 (**Fig. 2**, lanes 3) or from control plants infected with virus not harboring the Bet v 1 coding sequence (**Fig. 2**, lanes 4) were performed with individual sera from birch pollen-allergic patients and the standard serum pool. All individual sera as well as the standard serum pool displayed IgE binding to nBet v 1 present in birch pollen, to purified *E. coli* rBet v 1, and to Bet v 1 expressed in *N. benthamiana* (**Fig. 2**, lanes 1–3). No IgE binding could be detected to leaf protein extracts from control plants (**Fig. 2**, lanes 4). The serum pool of nonatopic donors did not show IgE binding to any proteins. Similarly, the buffer controls performed for an identical set of protein blots without the addition of sera remained negative (data not shown).

ELISA and ELISA cross-inhibition experiments

To compare the binding capacity to the monoclonal anti-nBet v 1 antibody BIP1 serial dilutions of nBet v

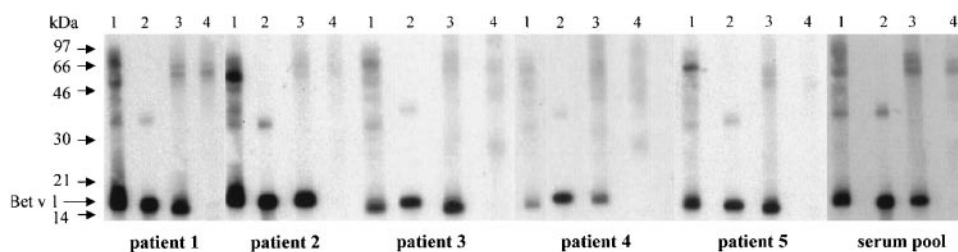


Figure 2. Immunoblot analysis of IgE binding of patients' sera with birch pollen allergy to nBet v 1, *E. coli* rBet v 1, tobacco rBet v 1 and to control plant extracts. Natural birch pollen extract (lanes 1), purified *E. coli* rBet v 1 (lanes 2), Bet v 1 present in crude protein extracts of *N. benthamiana* leaves infected with the viral construct (lanes 3), and extract of *N. benthamiana* leaves infected with the viral vector not harboring the Bet v 1 coding sequence (lanes 4) were blotted to nitrocellulose and probed with individual sera and a serum pool of birch pollen-allergic patients. IgE binding to Bet v 1 present in birch pollen extracts to purified *E. coli* rBet v 1 and to rBet v 1 expressed by the viral construct in *N. benthamiana* leaves was observed for all serum samples. Controls were carried out with a normal human serum pool (NHS) and with buffer instead of serum under identical conditions (data not shown).

1, tobacco rBet v 1 and *E. coli* rBet v 1 were tested in ELISA experiments. For each antigen concentration, four determinations were performed. Mean values are given in **Fig. 3**. Detection was carried out using polyclonal rabbit anti-Bet v 1 IgG and peroxidase-conjugated mouse anti-rabbit IgG antibodies. At all evaluated protein concentrations, a comparable binding capacity was observed for each preparation of Bet v 1 in a dose-dependent manner (Fig. 3). No specific binding was observed in the controls (data not shown).

Using the standard serum pool of birch pollen-allergic patients, cross-inhibition was determined by ELISA experiments. Natural Bet v 1 and tobacco rBet v 1 had the same inhibitory effect on IgE binding to nBet v 1 (**Fig. 4A**) and to tobacco rBet v 1 (Fig. 4B), respectively, demonstrating that the IgE binding epitopes of the two proteins are equivalent. Amounts as low as 2.1 $\mu\text{g}/\text{ml}$ for nBet v 1 and 2.3 $\mu\text{g}/\text{ml}$ for tobacco rBet v 1 resulted in 50% inhibition of IgE binding nBet v 1 (Fig. 4A), whereas 50% inhibition of IgE binding to tobacco rBet v 1 was achieved by a concentration of 2.5 $\mu\text{g}/\text{ml}$ tobacco rBet v 1 or 3.1 $\mu\text{g}/\text{ml}$ nBet v 1. Both proteins were able to fully inhibit the IgE binding to solid-phase bound nBet v 1 or tobacco rBet v 1. No inhibition of patients' IgE binding to nBet v 1 or tobacco rBet v 1 was observed when sera were preincubated with BSA (Fig. 4A, B).

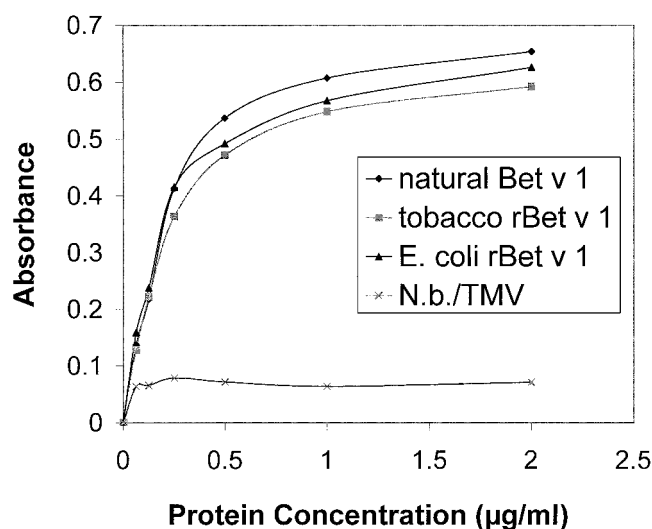


Figure 3. Quantitative analysis of Bet v 1 binding to the monoclonal anti-nBet v 1 antibody BIP1. Serial dilutions of purified nBet v 1 (◆), *E. coli* rBet v 1 (▲), and rBet v 1 from protein extracts of *N. benthamiana* leaves infected with the viral construct (■) were compared in an ELISA assay for their binding capacity to the mouse monoclonal anti-nBet v 1 antibody BIP1. Protein extracts from *N. benthamiana* leaves infected with the virus without the Bet v 1 coding sequence (x) were used as controls. Bet v 1 bound to BIP1 was detected with a polyclonal rabbit anti-nBet v 1 antiserum.

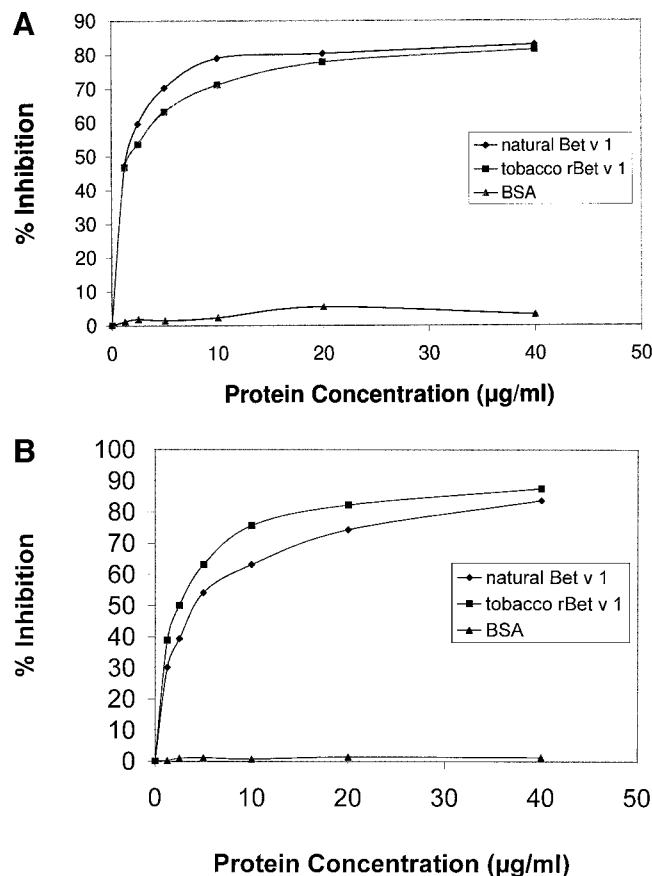


Figure 4. Inhibition of IgE binding to solid-phase coated nBet v 1 from birch pollen (A) or to solid-phase coated rBet v 1 transiently expressed in *N. benthamiana* leaves (B). A serum pool of birch pollen-allergic patients was preincubated with different amounts of nBet v 1 (◆), tobacco rBet v 1 (■), or BSA (▲) as negative control. Preincubated serum pool samples were transferred to wells coated with nBet v 1 (A) or tobacco rBet v 1 (B). Bound IgE was detected by antigen-specific ELISA.

Cutaneous type I hypersensitivity reaction in BALB/c mice and PCA

BALB/c mice immunized with purified rBet v 1 produced in *E. coli* [Fig. 5; rBet v 1/ $\text{Al}(\text{OH})_3$], crude *N. benthamiana* leaf extract containing Bet v 1 [Fig. 5; tBet v 1/ $\text{Al}(\text{OH})_3$], or crude birch pollen extract [Fig. 5; BP/ $\text{Al}(\text{OH})_3$] displayed equally strong immediate type skin reactions 20 min after intradermal injection of *E. coli* rBet v 1 (Fig. 5, lower right quadrants) or birch pollen extract (Fig. 5, lower left quadrants) in comparison to the positive control, the mast cell degranulation compound 48/80 (Fig. 5, upper left quadrants). No allergen-specific skin reactions were elicited in the control mice immunized with crude leaf extract from *N. benthamiana* plants infected with the viral vector without the Bet v 1 coding sequence [Fig. 5; *N.b.*/ $\text{Al}(\text{OH})_3$]. Injections with PBS alone did not elicit a skin reaction in any of the animals (Fig. 5, upper right quadrants). To confirm that the type I skin reactions were due to

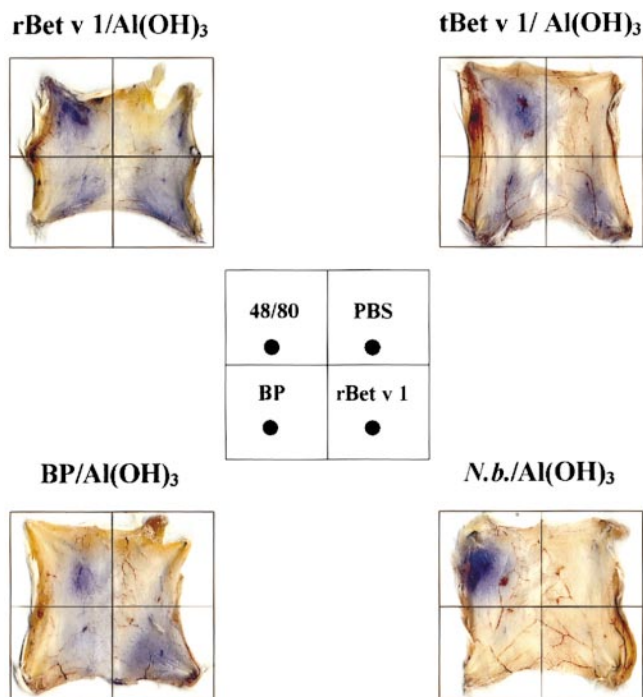


Figure 5. Representative skin sections of type I skin tests of mice immunized three times i.p. with $\text{Al}(\text{OH})_3$ adsorbed purified rBet v 1 produced in *E. coli* (rBet v 1, upper left panel), crude *N. benthamiana* leaf extract containing Bet v 1 (tBet v 1, upper right panel), crude birch pollen extract (BP, lower left panel), or crude extract from *N. benthamiana* leaves infected with the viral vector without the Bet v 1 coding sequence (*N.b.*, lower right panel). Twenty minutes after intradermal injection of the mast cell degranulation compound 48/80 (20 $\mu\text{g}/\text{ml}$, upper left quadrants), PBS (upper right quadrants), birch pollen extract (2.5 $\mu\text{g}/\text{ml}$, lower left quadrants), or *E. coli* rBet v 1 (2.5 $\mu\text{g}/\text{ml}$, lower right quadrants) the skin reaction was measured at the inside of the abdominal skin.

allergen-specific IgE antibodies and not to allergen-specific IgG1, PCA reactions were performed in Wistar rats using the immune sera of the BALB/c mice. The size of the reaction elicited by injection of tobacco rBet v 1 immune sera (1:4 dilution) into the rats was 89% of that elicited by the *E. coli* rBet v 1 immune sera. The color intensity of both reactions was comparable. The 1:20 dilution of both mice immune serum pools induced only weak to negative reactions. The PCA results underline that the type I skin tests were mediated by comparable allergen-specific IgE antibody levels.

Allergen-specific antibody levels in sera of BALB/c mice

In accordance with the type I skin tests and PCA, allergen-specific IgE antibodies, determined by ELISA, were equally high in mice immunized with purified *E. coli* rBet v 1 ($\text{OD}_{405}=0.6\pm0.16$) and nonpurified tobacco rBet v 1 ($\text{OD}_{405}=0.6\pm0.17$). IgG1 antibody levels were slightly higher in the *E. coli*

rBet v 1-immunized mice ($\text{OD}_{405}=2.3\pm0.4$) than in tobacco rBet v 1-immunized mice (1.01 ± 0.13), but the difference was not statistically significant. IgG2a antibody levels were low in either of the groups (*E. coli* rBet v 1: $\text{OD}_{405}=0.06\pm0.08$, tobacco rBet v 1: $\text{OD}_{405}=0.01\pm0.17$). Thus, immunization with *E. coli* rBet v 1 or tobacco rBet v 1 led to a typical Th2-like immune response *in vivo* and *in vitro*. The immune response after the immunization with BP extract also displayed a Th2 profile (IgE: $\text{OD}_{405}=0.29\pm0.15$; IgG1: $\text{OD}_{405}=1.75\pm0.01$; IgG2a: $\text{OD}_{405}=0.04\pm0.1$). Immunization with extracts from *N. benthamiana* plants not expressing Bet v 1 did not result in allergen-specific antibody responses as already observed in the type I skin tests *in vivo*. Baseline OD_{405} units of preimmune sera from all animals were 0.156 ± 0.012 for IgG1, 0.246 ± 0.029 for IgG2a, and 0.166 ± 0.014 for IgE. The values reported above are those from the immune sera after subtraction of the baseline values.

DISCUSSION

In this study we have used a plant viral vector to rapidly express the clinically relevant birch pollen allergen Bet v 1 in the tobacco-related species *N. benthamiana*. The recombinant allergen was accumulated to 0.6% of total soluble protein in leaves. The use of human sera from birch pollen-allergic patients for Western blot and ELISA cross-inhibition experiments indicated that no differences in IgE epitopes existed between tobacco rBet v 1 and nBet v 1 present in birch pollen. Moreover, immunizing BALB/c mice with plant extracts containing Bet v 1 elicited immune responses equivalent to the purified allergen produced in *E. coli* as well as to natural Bet v 1 present in birch pollen extracts.

There is a growing need for the experimental, diagnostic, and clinical use of pure allergens (2–4). Based on the detailed information available on the birch pollen allergen Bet v 1 (5–16), this protein was chosen as a model allergen to assess the possibilities of transient expression in plants infected by recombinant viral vectors. As isolation from natural sources is exceedingly time and cost intensive, allergens are conventionally produced as recombinant proteins in *E. coli*. By comparison, few examples for allergen expression in yeast have been published (19–24). Recombinant production of properly folded proteins in plants represents a major advantage over the *E. coli* system, where recombinant proteins often have to be refolded by denaturation/renaturation. In many cases only a small fraction of the protein adopts the correct conformation. It is generally assumed that the expression of plant allergens in a homologous system poses fewer problems regarding

the correct folding and solubility of recombinant proteins. In addition, plant-expressed recombinant proteins do not require extensive purification from bacterial toxins, making production less time and cost intensive.

It has been demonstrated that transgenic plants that express foreign proteins with pharmaceutical value represent a feasible alternative to fermentation-based production systems. *Agrobacterium*-mediated transformation has been successfully applied for production of several subunit vaccines (25–28). Mice fed with extracts or edible tissue of these plants developed both serum and secretory antibodies specific to the recombinant antigen (25–28). However, to obtain genetically engineered plants after *Agrobacterium*-mediated transformation is a very time-consuming process and the quantity of the heterologous protein expressed does not always reach satisfactory levels.

In contrast to conventional plant transgenic approaches, a second strategy for the transient expression of foreign proteins in plants has been developed using viral vectors. Biologically active α -trichosanthin and epitopes with immunoprotective properties have been produced in tobacco plants transfected by recombinant TMV (30, 33, 34). Very recently, the production of a tumor-specific vaccine in *N. benthamiana* plants has been reported using a TMV-based expression system (31). The expression and application of a single-chain variable region of an Ig of a B cell lymphoma clearly demonstrated the speed and the efficiency of this system (31). We have applied for the first time plant virus-directed transient expression to produce an allergen in the tobacco-related species *N. benthamiana*. It was our aim to establish and evaluate the suitability of such a plant-based expression system for the production of plant-derived allergens as an alternative to *E. coli* or yeast-based production systems. Therefore, it was required that the plant-expressed heterologous allergen displayed immunological characteristics equivalent to the natural counterpart.

We used a TMV vector to transiently express the allergen Bet v 1 by placing its coding sequence under the control of the viral coat protein subgenomic promoter (29). We were able to demonstrate the expression of Bet v 1 in leaves, stems, and roots of infected *N. benthamiana* plants (Fig. 1). In leaves, Bet v 1 was accumulated to 2.525% of the total soluble protein, which is slightly higher as reported for the TMV-directed expression of α -trichosanthin (33). These findings show that this system is capable of producing high amounts of recombinant protein, exceeding those of transgenic plants. Levels of 0.23% were reported for expression of the Norwalk virus capsid protein in transgenic tobacco leaves and 0.3% for the cholera toxin B subunit in potato

microtubers (26, 28). Bet v 1 expression in *N. benthamiana* by far exceeds the 0.01% yield found for the hepatitis B virus surface antigen or for *E. coli* LT-B in transgenic tobacco or potato (25, 27).

Binding of IgE antibodies to Bet v 1 is strictly related to the molecule's conformation. Slight changes in the conformation of Bet v 1 significantly lower its capacity to bind IgE (35). More severe alterations of the molecule's structure completely abolish its IgE binding capability (35). We used several individual sera and a standard serum pool of birch pollen-allergic patients' sera to cover the spectrum of IgE antibodies induced in atopics as a response to the sensitization against Bet v 1. The IgE binding capacities of Bet v 1 from birch pollen, rBet v 1 from *E. coli*, and rBet v 1 from *N. benthamiana* were equivalent (Fig. 2). No detectable cross-reactive protein was observed in the 17 kDa range in the extracts of *N. benthamiana* control plants infected with the viral vector without the Bet v 1 coding sequence (Fig. 2, lanes 4). Cross-reactive allergens in the higher molecular mass ranges as observed in the immunoblots have been detected in a wide range of plant species and are assumed to be homologous conserved proteins. Their identity is still under investigation.

In addition to the immunoblot studies, we have used ELISA and ELISA inhibition assays to evaluate the immunological characteristics of Bet v 1 produced in *N. benthamiana* plants. Comparable binding properties to the anti-nBet v 1 monoclonal antibody BIP1 were observed for natural Bet v 1 purified from birch pollen, Bet v 1 expressed in *N. benthamiana*, and Bet v 1 produced in *E. coli*. This result confirms that the BIP1 epitope is well preserved during the expression of Bet v 1 in *N. benthamiana* (Fig. 3). Moreover, patients' sera preadsorbed with either nBet v 1 or tobacco rBet v 1 lost their IgE reactivity to nBet v 1 (Fig. 4A) as well as to tobacco rBet v 1 (Fig. 4B) in a dose-dependent and comparable manner. From these results, we conclude that the IgE binding properties of the tobacco Bet v 1 are equivalent to Bet v 1 present in birch pollen. These cross-inhibition experiments further indicate that the conformation of Bet v 1 produced in *N. benthamiana* is equivalent to the Bet v 1 from birch pollen, as even small changes in the overall 3-dimensional structure abolish IgE binding reactivity (35, 36).

We had previously established a mouse model for type I allergy to birch pollen. The immunization protocols used in this model led to increased IgE/IgG1 vs. low IgG2a antibody levels and positive skin tests *in vivo*, thus representing an immune response comparable to that of human type I allergy (14, 37, 38). Consequently, this mouse model is a useful tool to evaluate and characterize new systems for recombinant allergen production. Previous studies have

shown that no significant immunological differences between purified *E. coli* rBet v 1 and natural Bet v 1 from birch pollen exist (6, 15, 38). We have applied this mouse model to compare immune responses after immunization with standard purified rBet v 1 produced in *E. coli* to Bet v 1 produced in *N. benthamiana* plants. Notably, the application of Bet v 1 in a total plant protein extract (i.e., nonpurified) induced immune responses that were equal in terms of antibody production profile as well as skin test reactivity to those after immunization with purified *E. coli* rBet v 1. We have further demonstrated that no antibody responses or positive skin reactivity to the allergen were induced after immunization with the crude extract from *N. benthamiana* plants infected with the TMV vector not carrying the Bet v 1 coding sequence.

Low toxicity, solubility, and production of high amounts of correctly folded recombinant proteins at low cost are prerequisites for developing new vaccination strategies. Vaccines against several infectious diseases have already been produced in transgenic plants (25–28). In particular, production of oral vaccines in edible plant tissues expressing subunit vaccines has shown the feasibility of this concept (25–28). Only recently the concept of oral tolerance or oral vaccination has also gained interest for the treatment of allergic diseases (39–41). The required doses of recombinant allergens for oral applications are substantial and therefore expensive. Using the TMV-directed transient expression system, the above-mentioned requirements can be met. In any case, this expression system, which offers a low cost means of producing allergens in plants, will support a broad range of applications in the field of molecular allergology. FJ

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