Molecular characterization of recombinant T1, a non-allergenic periwinkle (*Catharanthus roseus*) protein, with sequence similarity to the Bet v 1 plant allergen family

Sylvia LAFFER*, Said HAMDI†, Christian LUPINEK*, Wolfgang R. SPERR‡, Peter VALENT‡, Petra VERDINO§, Walter KELLER§, Monika GROTE‖, Karin HOFFMANN-SOMMERGRUBER*, Otto SCHEINER*, Dietrich KRAFT*, Marc RIDEAU¶ and Rudolf VALENTA*[1]

*Department of Pathophysiology, University of Vienna, Währinger Gürtel 18-20, A-1090 Vienna, Austria, †Biologie Cellulaire et Moleculaire du Développement des Plantes, Université de Bordeaux, Avenue des Facultés, F-33405 Talence, France, ‡Division of Haematology and Haemostaseology, Department of Internal Medicine I, University of Vienna, Währinger Gürtel 18-20, A-1090 Vienna, Austria, §Institute of Chemistry, Structural Biology, Karl-Franzens-University, Heinrichstr. 28, A-8010 Graz, Austria, ‖Institute of Medical Physics, University of Münster, Robert-Koch-Str. 33, Münster, Germany, and ¶Laboratoire de Biologie Moléculaire et Biochimie Végétale, Faculté de Pharmacie, EA 2106, 31, Avenue Monge, F-37200 Tours, France

More than 25 % of the population suffer from Type I allergy, an IgE-mediated hypersensitivity disease. Allergens with homology to the major birch (*Betula verrucosa*) pollen allergen, Bet v 1, belong to the most potent elicitors of IgE-mediated allergies. T1, a cytokinin-inducible cytoplasmic periwinkle (*Catharanthus roseus*) protein, with significant sequence similarity to members of the Bet v 1 plant allergen family, was expressed in *Escherichia coli*. Recombinant T1 (rT1) did not react with IgE antibodies from allergic patients, and failed to induce basophil histamine release and immediate-type skin reactions in Bet v 1-allergic patients. Antibodies raised against purified rT1 could be used for *in situ* localization of natural T1 by immunogold electron microscopy, but did not cross-react with most of the Bet v 1-related allergens. CD analysis showed significant differences regarding secondary structure and thermal denaturation behaviour between rT1 and recombinant Bet v 1, suggesting that these structural differences are responsible for the different allergenicity of the proteins. T1 represents a non-allergenic member of the Bet v 1 family that may be used to study structural requirements of allergenicity and to engineer hypo-allergenic plants by replacing Bet v 1-related allergens for primary prevention of allergy.

Key words: allergy, cross-reactivity, recombinant periwinkle protein, pathogenesis-related proteins.

INTRODUCTION

We have previously identified in periwinkle (*Catharanthus roseus*) cells a cytokinin-inducible plant protein, designated T1 [1]. The cDNA and deduced amino acid sequence of T1 show significant sequence identity with the major birch (*Betula verrucosa*) pollen allergen, Bet v 1, and thus could be identified as a member of the Bet v 1 allergen family [1,2]. More than 100 million allergic patients are sensitized against the Bet v 1 allergen family [2,3]. The cDNA and deduced amino acid sequence of Bet v 1 showed significant sequence homology to several pathogenesis-related plant proteins which are activated in plants after infection and wounding [2]. cDNAs coding for Bet v 1-related allergens were isolated from pollens of several trees [e.g. alder (*Alnus glutinosa*), hazel (*Corylus avellana*), hornbeam (*Carpinus betulus*) and chestnut (*Castanea dentata*)] [4–7] and from plant-derived food (fruits and vegetables) [8,9]. The deduced amino acid sequences of recombinant T1 (rT1) and of the members of the Bet v 1 allergen family show an overall sequence identity of between 35–85 %. In addition all these proteins contain a highly conserved P-loop motif, which also occurs in nucleotide-binding proteins [10]. The recent analysis of the structure of the Bet v 1-related allergen from cherry (*Prunus avium*), Pru av 1, and a Bet v 1 isoform provided evidence that Bet v 1-related proteins may represent steroid carriers [11,12]. Allergic patients show extensive IgE antibody cross-reactivity with members of the Bet v 1 allergen family [8].

Previously, we analysed two-dimensional-gel-separated periwinkle cell extracts and found that natural T1, a Bet v 1-homologous protein, did not react with IgE antibodies of Bet v 1-allergic patients [1]. In order to study the allergenic activity of rT1 in detail we expressed rT1 in *Escherichia coli* and purified the recombinant protein to homogeneity. Secondary structure contents, fold of rT1 and the presence of Bet v 1-cross-reactive epitopes were investigated. The allergenic activity of purified rT1 and rBet v 1 were compared by measuring basophil histamine release and by skin testing in Bet v 1-allergic patients. In addition, we investigated the immunogenic activity of rT1 by testing rT1-induced antibodies for reactivity with natural and recombinant T1, as well as with Bet v 1-related allergens.

We discuss our findings that rT1 lacked allergenic activity and did not induce a cross-reactive immune responses to Bet v 1-related allergens regarding the possibility of replacing Bet v 1-related plant allergens by the non-allergenic T1 protein in order to create hypo-allergenic plant products for primary prevention of allergy.

EXPERIMENTAL

Sequence comparison and alignments of rT1 and representative members of the Bet v 1 allergen family

The deduced amino acid sequence of rT1 was compared with the sequences deposited in the National Institutes of Health databases

Abbreviations used: TFA, trifluoroacetic acid; the prefix r denotes recombinant (e.g. rT1).

[1] To whom correspondence should be addressed (e-mail Rudolf.Valenta@akh-wien.ac.at).

Table 1 Demographic, clinical and serological characterization of birch pollen allergic patients and a non-allergic individual

Sex (F, female; M, male), age and allergen sources (t, tree pollen; g, grass pollen; w, weed pollen; f, food; mi, mites; a, animals) are displayed. Type of symptoms (r, rhinitis; c, conjunctivitis; ad, atopic dermatitis; as, asthma) and treatment (it, immunotherapy; ah, antihistamines; co, corticosteroids) are also indicated. Total IgE levels (k-units/l), and specific IgE levels (k-units of antigen/l) to birch pollen allergens and rBet v 1 were determined by CAP RAST measurements. In addition, IgE reactivities to rBet v 1 and rT1 were determined by ELISA (*A*, absorbance).

Individual	Sex	Age (years)	Allergies	Symptoms	Treatment	Total IgE (k-units/l)	IgE to Birch (k-units of antigen/l)	IgE to rBet v 1 (k-units of antigen/l)	IgE reactivity (*A*)	
									rBet v 1	rT1
1	F	27	t,g,w,f	r,c	—	124	41.7	48.5	0.67	0.094
2	M	61	t,g,w,f	r,c	it	36.9	26.4	23	0.961	0.060
3	F	26	t,g,w,f,mi,a	r,c	ah	278	54.5	26.1	0.541	0.061
4	M	36	t,g,f,mi	r,c,ad	it,co	81.9	11.9	11.5	0.191	0.062
5	F	40	t,a,f	r,c,as	it,ah,co	267	79.8	74.8	1.067	0.072
6	F	49	t,a	r,c	ah	178	17.5	13.2	0.35	0.067
7	M	54	t,mi	r,c	it,co	152	8.85	2.69	0.27	0.06
8	M	54	t,g	r,c	it,co	125	51.3	22.5	0.296	0.064
9	M	38	t,g,w	r,c,as	ah	50	13.7	19.2	0.197	0.048
10	M	28	t,g,w	r,c	it	41	0.5	< 0.35	0.047	0.069
11	M	52	t,g,a	r,c	ah	1246	17.1	< 0.35	0.057	0.075
12	F	35	—	—	—	33	< 0.35	< 0.35	0.07	0.072

(GenBank®, SwissProt, PIR Protein). Related allergens were retrieved using the BLASTP program and grouped according to sequence homology. The sequences of rT1 and members of the Bet v 1 allergen family were compared with each other using the GAP program (Wisconsin Package) and the percentage sequence identity was expressed. Potential glycosylation sites and sequence motifs were identified with the MacVector program (Kodak).

Characterization of allergic patients

Birch pollen allergic patients exhibiting allergic symptoms (rhinitis, conjunctivitis or asthma) during birch pollen season were included in the present study. The diagnosis of birch pollen allergy was confirmed by skin-prick testing and determination of specific IgE antibodies (CAP RAST, Pharmacia Diagnostics, Uppsala, Sweden). The demographic, clinical and serological characterization of the birch pollen allergic patients and a non-allergic control individual are summarized in Table 1.

Characterization of antibodies and recombinant allergens

Generation and characterization of mouse monoclonal antibodies 0590 and 0639 specific for Bet v 1 [13], and of mouse monoclonal anti-Bet v 1 antibody Bip 1 [14,15] have been reported previously. The mouse monoclonal antibody 4A6 [16] was raised against purified recombinant birch profilin.

Recombinant allergens from birch pollen (rBet v 1), celery (*Apium graveolens*) (rApi g 1), apple (*Malus domestica*) (rMal d 1) and hazel pollen (rCor a 1) were expressed in *E. coli* and purified as described previously [17,18] (Biomay, Vienna, Austria). Recombinant carrot (*Daucus carota*) allergen (rDau c 1) was expressed in *E. coli* and purified as described previously [19].

A rabbit anti-T1 antiserum was generated by immunization of a rabbit with purified rT1 using complete and incomplete Freund's adjuvants (Charles River, Kisslegg, Germany).

Expression and purification of rT1

Biological source, and isolation of RNA and cDNA coding for T1 were described previously [1]. The cDNA, containing the coding sequence of rT1, was obtained by PCR amplification using the following primers: forward and reverse primer contained an *Nde*I (underlined) and *Bam*HI (*italics*) restriction site respectively: 5′-GGGTGTTATTTCTTATCATATGGGAG-3′ (corresponding to the N-terminal end of T1); 5′-GAAAATAG-AG*GGATCC*GATTTTGTTA-3′ (corresponding to the C-terminal end of T1). The PCR product was cut with *Nde*I and *Bam*HI, gel-purified and subcloned into the *Nde*I and *Bam*HI sites of the expression plasmid pET 17b (Novagen, Madison, WI, U.S.A.) to obtain a plasmid expressing T1 as a non-fusion protein without tag. The DNA sequence of the construct was confirmed by sequencing of both DNA strands [20]. Plasmid pET 17b containing the T1 cDNA was transformed into *E. coli* BL21 (DE3) (Novagen). Freshly transformed bacteria were grown in Luria–Bertani medium containing 100 mg/l ampicillin to a D_{600} of 0.4. The expression of the recombinant protein was induced by addition of isopropyl β-D-thiogalactoside to a final concentration of 1 mM and growing the bacteria for additional 3 h. Since rT1 accumulated in the insoluble pellet fraction of the bacteria, rT1 was purified using an inclusion body preparation protocol: bacteria were harvested by centrifugation and were homogenized in 25 mM imidazole, pH 7.4, 0.1 % (v/v) Triton X-100 (5 ml/g of wet mass cells after centrifugation) with an Ultraturrax (IKA, Stauffen, Germany). DNA was digested by addition of DNase I (100 μg/g of wet mass cells after centrifugation) and stirring for additional 5 min at 20 °C. The bacterial extract was then centrifuged (8000 ***g***, 4 °C, 30 min) and the pellet was resuspended in 50 mM Tris, pH 8.0 (2 ml/g of wet mass cells after centrifugation), and then buffer A (20 mM Tris, pH 8.0, 0.2 M NaCl, 1 % deoxycholate, 1 mM 2-mercaptoethanol) (4 ml/g of wet mass cells after centrifugation) was added. The inclusion body-containing mixture was stirred at 20 °C for 10 min and was then centrifuged at 4 °C (6000 ***g***, 15 min). The inclusion body-containing pellet was resuspended in four-fold diluted buffer A, and the inclusion bodies were washed four times using this buffer and once in 10 mM Tris, pH 8.0, 3 % isopropanol (5 ml/g of wet mass cells after centrifugation). Washed inclusion bodies were harvested by centrifugation (6000 ***g***, 4 °C, 15 min) and solubilized in 8 M urea, 10 mM Tris, pH 8.0, 1 mM EDTA, 5 mM 2-mercaptoethanol at 20 °C overnight. The homogenate was diluted with 10 mM Tris, pH 8.0, to a final concentration of 6 M urea and centrifuged (8000 ***g***, 4 °C, 30 min) to remove insoluble materials. The supernatant, which contained rT1, was then dialysed back against reduced concentrations of urea (4 M urea, 2 M urea) and finally against 5 mM Tris/HCl, pH 8.0. Certain

rT1 preparations were further purified by reversed-phase HPLC (Vydac 218TPB column; 5 μm, 300 Å, 150 mm × 4 mm). The gradient consisted of solvents A [0.1 % triflouroacetic acid (TFA) in water], and B (0.1 % TFA in acetonitrile), and was extended from 10 % B to 90 % B in 15 min. The flow rate was 1 ml/min. All rT1 preparations were checked for purity by SDS/PAGE and isoelectric focusing.

MS and CD analysis of rT1

Laser desorption mass spectra were acquired in a linear mode by a matrix-assisted laser-desorption ionization–time-of-flight instrument (Compact MALDI II; Kratos, Manchester, U.K.), operating at 20 kV acceleration voltage, and equipped with a nitrogen UV laser (337 nm, pulse duration 3 s) (piCHEM Research and Development, Graz, Austria). Samples were dissolved in 10 % acetonitrile (0.1 % TFA), α-cyano-4-hydroxycinnamic acid was used as a matrix dissolved in 60 % acetonitrile (0.1 % TFA). For sample preparation, a 1:1 mixture of protein solution and matrix solution was deposited on to the target and air dried.

CD measurements were performed in double-distilled water with protein concentrations of 0.25 g/l for both proteins (1.55×10^{-5} M rT1 and 1.42×10^{-5} M rBet v 1). The CD investigations were carried out in a Jasco J-715 spectropolarimeter using a 0.1-cm pathlength cell with cooling jacket connected to a water thermostatic device. The samples were heated to 95 °C at a rate of 1 °C/min and cooled down using the same parameters. A continuous temperature scan was performed at 212 nm with a step resolution of 0.5 °C and a wait time of 1 min. Spectra were recorded at 20, 30, 40, 50, 60, 70, 80, 90 and 95 °C with 0.5 nm resolution at a scan speed of 50 nm/min and resulted from averaging 5 scans. The final spectra were baseline-corrected by subtracting the corresponding double-distilled water spectra obtained under identical conditions. Results were expressed as the mean residue ellipticity [θ] at a given wavelength. The data were fitted with the secondary structure estimation program DicroProt [21] according to Yang et al. [22].

ELISA

Purified rT1 or purified rBet v 1 were dissolved in a coating buffer (0.1 M $NaHCO_3$, pH 9.6), each at a concentration of 10 μg/ml and were coated on to ELISA plates (Nunc, Roskilde, Denmark) overnight at 4 °C. Plates were washed five times with Tris-buffered saline, pH 7.5/0.05 % (v/v) Tween-20 (TBST) and were blocked for 2.5 h at 37 °C with TBST containing 1 % BSA. The ELISA plate-bound proteins were incubated overnight at 4 °C with 1:10 in TBST-diluted sera from Bet v 1-allergic patients and, for control purposes, with serum from a birch pollen allergic patient without reactivity to rBet v 1, as well as with a serum from a non-allergic individual. The successful coating of rT1 to the ELISA plate was demonstrated in a parallel experiment with a rabbit anti-T1 antiserum, diluted 1:1000 in TBST. Bound human IgE was detected with an alkaline phosphatase-conjugated mouse monoclonal anti-human IgE antibody (PharMingen, San Diego, CA, U.S.A.). Bound rabbit antibodies were detected with alkaline phosphatase-conjugated goat anti-rabbit IgG (H + L) antibodies (Dianova, Hamburg, Germany).

Immunoblots

Periwinkle callus cells were cultured on a 2,4-dichlorophenoxyacetate-free medium containing 5 μM zeatin to induce the accumulation of T1 [1]. Total proteins were extracted from 15-day-old tissues. Purified rT1 or purified recombinant allergens (5μg/cm of gel) or periwinkle callus culture extract were separated by 12.5 % SDS/PAGE [23] and blotted on to nitrocellulose (Schleicher & Schuell, Dassel, Germany) [24]. Nitrocellulose strips were blocked in buffer B [50 mM sodium phosphate, pH 7.5, 0.5 % (w/v) BSA, 0.5 % (v/v) Tween-20, 0.05 % NaN_3] twice for 10 min and once for 30 min. Membranes were incubated with human sera, diluted 1:10 in buffer B, or with rabbit or mouse antibodies, diluted 1:1000 in buffer B. Bound human IgE antibodies were detected with 1:10 diluted ^{125}I-labelled anti-human IgE antibodies (Pharmacia Diagnostics), bound rabbit antibodies were detected with a 1:1000 diluted ^{125}I-labelled donkey anti-rabbit antiserum (Amersham, Little Chalfont, Bucks., U.K.) and bound mouse monoclonal antibodies with a 1:1000 diluted ^{125}I-labelled sheep anti-mouse antiserum (Amersham) and visualized by autoradiography (Kodak XOMAT film; Kodak, Heidelberg, Germany).

Histamine-release experiments

Histamine-release experiments were performed with peripheral blood polymorphonuclear leucocytes, which were obtained by dextran sedimentation from Bet v 1-allergic patients [25]. Cells were incubated with increasing concentrations (0.001, 0.01, 0.1, 1 and 10 μg/ml) of purified rT1 and for control purposes with rBet v 1 at 37 °C for 30 min. Histamine released into the cell-free supernatant was measured by RIA (Immunotech, Marseille, France), and expressed as percentage of total histamine determined after cell lysis. Results are displayed as mean values of duplicate determinations.

Skin-prick testing

Allergic patients and control individuals were skin-prick tested with 20 μl aliqouts of test solution on their forearms [26]. The test solutions comprised rT1 and rBet v 1, which were diluted in double-distilled water at various concentrations (0.2, 1, 5, 25 and 125 μg/ml). Each individual was tested with commercially available birch pollen extract, with histamine (1 mg/ml) (ALK, Horsholm, Denmark) and with double-distilled water. Results were recorded 20 min after testing by photography and by transfering the ball-point pen marked wheal area with a scotch tape to paper. The mean wheal diameter (D_m) was calculated after measuring the maximal transversal (D) and horizontal (d) diameters using the following equation:

$$D_m = (D + d)/2$$

Immunogold electron microscopy

Cultured periwinkle cells, grown on a 2,4-dichlorophenoxyacetate-free medium containing zeatin, were fixed in buffered glutaraldehyde and embedded in Lowicryl K4M resin at −35 °C. Ultrathin sections were labelled with Protein G-purified rabbit anti-T1 antibodies, diluted 1:160 in PBS followed by 1:20 in PBS diluted goat anti-rabbit IgG antibodies coupled to 10 nm colloidal gold particles (British BioCell, Plano, Wetzlar, Germany). Controls were performed using Protein G-purified rabbit pre-immune antibodies instead of the purified anti-T1 antibodies and by incubating the sections with the gold-conjugated antibodies alone. Sections were stained with uranyl acetate. The sections were analysed in a Hitachi H500 transmission electron microscope (Hitachi Ltd, Japan).

Figure 1 Alignment of the deduced amino acid sequence of rT1 with rBet v 1 isoforms and Bet v 1-related allergens

The deduced amino acid sequence of rT1 was compared with three Bet v 1 isoforms (Bet v 1a, Bet v 1d and Bet v 1l), and with the major allergens from hazel pollen (Cor a 1), apple (Mal d 1), celery (Api g 1) and carrot (Dau c 1). Amino acids identical to T1 are indicated by hyphens, and points represent gaps induced for fit. Regions of high sequence similarity are boxed (white, α-helix 1; light grey, glycine-rich loop). Potential glycosylation sites are boxed in dark grey.

RESULTS

Sequence identity of rT1 with Bet v 1 isoforms and Bet v 1 cross-reactive allergens

The deduced amino acid sequence of T1 shows comparable and significant sequence identities with the major birch pollen allergen, Bet v 1 (40.4%), with Bet v 1-cross-reactive tree pollen allergens (alder Aln g 1, 41.7%; hazel Cor a 1, 41%; hornbeam Car b 1, 41%) and with Bet v 1-cross-reactive plant food allergens (apple Mal d 1, 41%; celery Api g 1, 38.8%; carrot Dau c 1, 36.8%; cherry Pru av 1, 44.2%). Sequence alignments of rT1 with well characterized Bet v 1 isoforms and allergens with proven cross-reactivity to Bet v 1 were performed [5,12,27–29]. This comparison of the deduced amino acid sequence of rT1 with those of three Bet v 1 isoforms (Bet v 1a, Bet v 1d, Bet v 1l), the major hazel pollen allergen, Cor a 1, and the major allergens from apple (Mal d 1), celery (Api g 1) and carrot (Dau c 1) is displayed in Figure 1. Two regions comprising nine or more amino acids with a sequence identity of at least 54% between all of the aligned proteins could be identified. They corresponded to a portion located in the first α-helix of Bet v 1 (Figure 1: boxed in white) and to a portion representing the conserved glycine-rich P-loop in Bet v 1-related proteins (Figure 1: boxed in light grey) [30]. Two potential glycosylation sites were predicted for T1 which were absent in the Bet v 1-cross-reactive allergens from apple, celery and carrot. In the three Bet v 1 isoforms and in the major hazel pollen allergen, Cor a 1, a glycosylation site was predicted which corresponded to the N-terminal glycosylation site of T1, whereas no glycosylation site corresponding to the C-terminal glycosylation site of T1 was found in the aligned Bet v 1-related allergens (Figure 1: boxed in dark grey). The recently published crystal structure of the Bet v 1l isoform [12] revealed the presence of a hydrophobic cavity capable of accommodating two hydrophobic steroid-like ligands. This cavity comprised an outer binding niche formed by amino acids which are neither conserved among Bet v 1-related allergens nor in T1. The amino acids forming the inner binding niche were conserved among Bet v 1-related allergens [12] and in T1.

Expression in *E. coli* and purification of rT1

Recombinant T1 was over-expressed in *E. coli* as a non-fusion protein without any tag and accounted for approx. 20% of the total *E. coli* proteins. Cell fractionation experiments showed that rT1 accumulated in the insoluble inclusion body fraction of the bacteria. Using an inclusion body preparation protocol, solubilization of the inclusion bodies in urea and slow dialysis against 5 mM Tris (pH 8.0) soluble pure rT1 could be obtained. Recombinant T1 was further purified to homogeneity by reverse-phase HPLC. Figure 2(A) shows the Coomassie Blue-stained SDS/PAGE gel containing pure rT1. The molecular mass of purified rT1 was determined by MS (16.17 kDa) which corresponded to the molecular mass calculated from the amino acid sequence of rT1 (Figure 2B).

Recombinant T1 is a folded protein with mixed α-helical and β-sheet conformation which differs from Bet v 1 with regard to secondary structure and thermal denaturation behaviour

Secondary structure predictions based on the amino acid sequences with PSIpred and PROF [31–33] resulted in nearly identical secondary structure contents for rT1 and Bet v 1a. In T1 a short N-terminal β-strand is followed by an α-helix, six adjacent β-strands and a long C-terminal α-helix (22% α-helix and 39.3% β-strand in total). The secondary structure of rBet v 1a is nearly identical, except for a slightly longer N-terminal β-strand followed by two α-helices (23.1% α-helix and 40.6% β-strand in total).

However, in contrast with the predictions, the CD spectroscopic comparison revealed significant differences between rT1 and

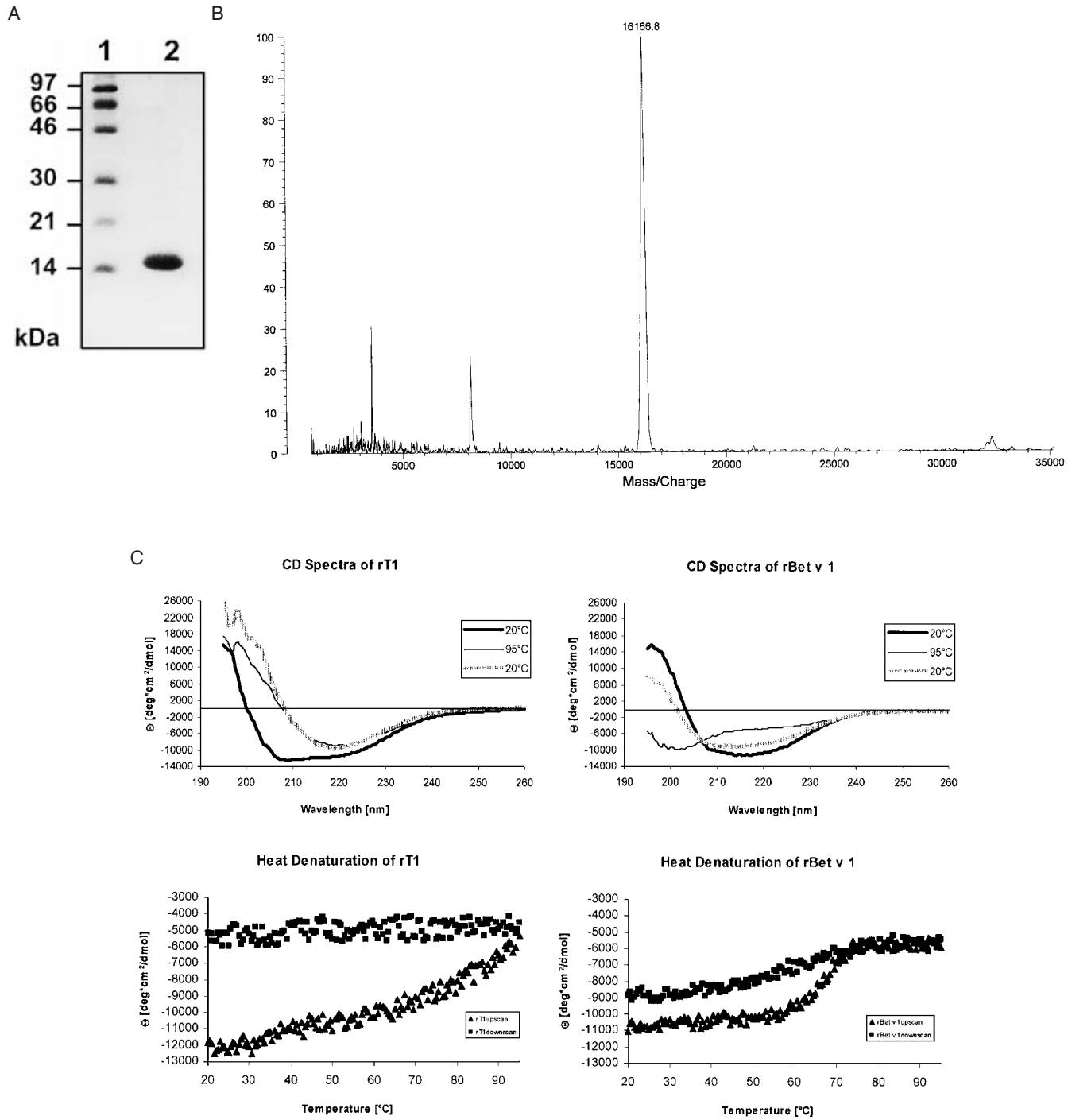

Figure 2 Coomassie-Blue staining, MS and far-UV CD analysis of rT1

(**A**) Coomassie Blue-stained SDS/PAGE gel. Lane 1, molecular mass markers; and lane 2, 5 μg of purified rT1. (**B**) MS analysis of purified rT1. The mass/charge ratio is shown on the *x*-axis. A peak at 16166.8 corresponds to the calculated molecular mass of the deduced amino acid sequence (16169.05).The signal intensity is displayed on the *y*-axis as the percentage of the most intense signal obtained in the investigated mass range. (**C**) Far-UV CD analysis of rT1 and rBet v 1: the mean residue ellipticity at 20 °C (continuous line, bold), at 95 °C (continuous line) and after cooling to 20 °C (broken line) is shown on the *y*-axis for different wavelengths. The protein concentration for rT1 was 1.55×10^{-5} M and for rBet v 1 was 42×10^{-5} M. Heat denaturation of rT1 and rBet v 1: the mean residue ellipticity at 212 nm (*y*-axis) was recorded during heating (upscan) and cooling (downscan).

rBet v 1. The 20 °C spectrum of rT1 is characterized by a minimum at 208 nm, a shoulder around 222 nm and a characteristic rise towards a maximum below 200 nm. The form of the spectrum indicates a mixed α/β-fold of the rT1 protein with higher amount of α-helical structure than predicted (Figure 2C). Secondary structure fitting procedures resulted in estimations of 36.5 % α-helix and 28.6 % β-strand for the rT1 protein. For the rBet v 1a protein a broad minimum at 215 nm and a maximum at 196 nm were observed, indicating a dominant β-sheet secondary structure content. The 20 °C CD spectrum of rBet v 1 is fitted with 24.6 % α-helix and 57.9 % β-sheet, which is in good accordance with the secondary structure elements observed in the crystal structure [30].

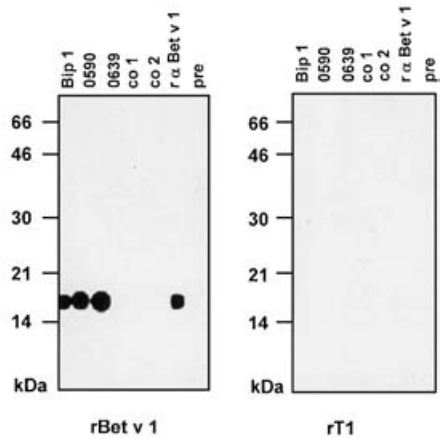

Figure 3 Bet v 1-specific antibodies do not react with nitrocellulose-blotted rT1

Bet v 1-specific monoclonal mouse antibodies Bip 1, 0590, 0639, an isotype-matched monoclonal antibody without specificity for Bet v 1 (lane co 1), buffer without addition of antibodies (lane co 2), a polyclonal rabbit anti-Bet v 1 antiserum (r α Bet v 1) and the corresponding pre-immune serum (pre) were exposed to nitrocellulose-blotted rBet v 1 and rT1.

The most significant differences between rT1 and rBet v 1 can be seen in the thermal denaturation experiment. rT1 changes continuously from the α/β-fold to a β-sheet-dominated structure upon heating to 95 °C. Upon cooling to 20 °C rT1 remains in this β-fold without any observable changes (Figure 2C). In contrast, the rBet v 1a protein shows a sharp sigmoidal unfolding transition to random-coil conformation with a melting point at 68 °C. The protein regains about 65 % of its initial β-sheet-dominated secondary structure upon cooling to 20 °C (Figure 2C).

Recombinant T1 does not react with Bet v 1-specific antibodies or serum IgE from Bet v 1-allergic patients

The presence of cross-reactive B-cell epitopes between rT1 and the major birch pollen allergen, rBet v 1, was tested with IgG antibodies raised against Bet v 1 and Bet v 1-specific IgE antibodies from birch pollen-allergic patients (Figure 3 and Table 1). Three monoclonal antibodies (Figure 3: Bip 1, 0590 and 0639) recognizing different epitopes on Bet v 1, and a rabbit anti-(Bet v 1) antiserum which cross-reacts with most of the Bet v 1-related allergens were tested for reactivity to rT1 and rBet v 1. The Bet v 1-specific mouse monoclonal antibodies and the rabbit anti-(Bet v 1) antiserum did not react with rT1, whereas rBet v 1 was strongly recognized (Figure 3). An isotype-matched mouse monoclonal antibody without specificity for Bet v 1, buffer without addition of primary antibodies and the pre-immune serum did not react with rBet v 1 or rT1 (Figure 3). These results show that Bet v 1-specific IgG antibodies do not recognize epitopes on rT1.

Next we investigated whether IgE antibodies of Bet v 1 allergic patients cross-react with rT1. Table 1 shows the lack of cross-reactivity of nine representative sera from Bet v 1-allergic patients with rT1. The nine patients who suffered from various birch pollen-induced manifestations of allergy (rhinoconjunctivitis, asthma and atopic dermatitis) contained different levels of total IgE, birch pollen extract- and rBet v 1-specific IgE (Table 1). All of the nine sera showed IgE reactivity to ELISA plate-bound rBet v 1, but no IgE binding to rT1 (Table 1). Two sera from birch pollen allergic patients without Bet v 1-specific IgE antibodies and one serum from a non-atopic individual, which were analysed for control purposes, showed no significant IgE reactivity to rBet v 1 or rT1 (Table 1, individuals 10–12). The successful coupling of rT1 to the ELISA plates was demonstrated by its reactivity with rabbit anti-T1 antibodies (results not shown).

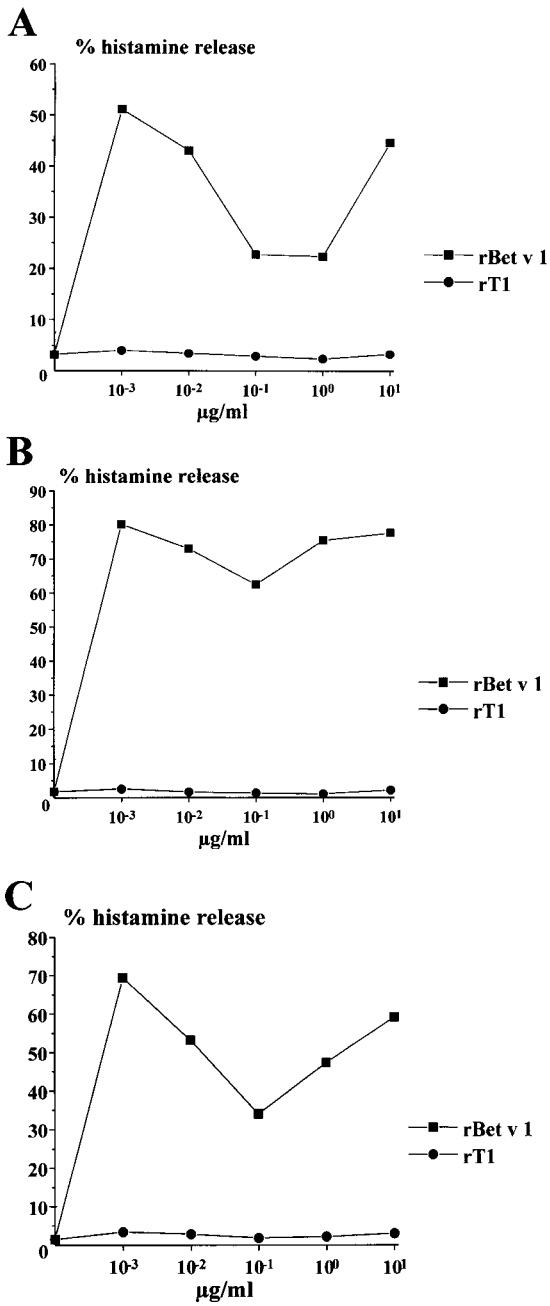

Figure 4 rT1 does not induce histamine release from basophils of Bet v 1-allergic patients

Basophils from three Bet v 1-allergic patients (**A**–**C**) were incubated with various doses of purified rT1 (●) or rBet v 1 (■). The percentage of histamine release is shown.

rT1 does not induce basophil histamine release or immediate-type skin reactions in Bet v 1-allergic patients

To investigate whether rT1 can induce allergic reactions in Bet v 1-sensitized patients, experiments were performed *in vitro* and *in vivo*. When granulocytes from three Bet v 1-allergic patients were incubated with various concentrations of purified rBet v 1 or rT1, rBet v 1 but not rT1 induced a dose-dependent release of histamine (Figure 4). In all three patients, maximal histamine

Table 2 rT1 does not induce immediate-type skin reactions in Bet v 1-allergic patients

Three Bet v 1-allergic patients (A–C), one grass-pollen allergic patient without Bet v 1-specific IgE antibodies (D) and a non-allergic person (E) were skin prick tested with different concentrations of rT1 and rBet v 1, as well as with birch pollen extract (Birch) and histamine (Histamine). The mean wheal diameters in mm are displayed. n.d., not determined.

	Mean wheal diameter (mm)											
	rT1 (μg/ml)					rBet v 1 (μg/ml)						
Individual	0.2	1	5	25	125	0.2	1	5	25	125	Birch	Histamine
A	0	0	0	0	0	2.5	5	7.5	8	n.d.	6.5	5.5
B	0	0	0	0	0	3	5	8.5	15	n.d.	8	3.5
C	0	0	0	0	0	3	4	6.5	7.5	n.d.	6	4
D	0	0	0	0	0	0	0	0	0	0	0	5
E	0	0	0	0	0	0	0	0	0	0	0	4.5

release was induced at 1 ng/ml of rBet v 1, whereas rT1 failed to induce histamine release up to a concentration of 10 μg/ml (Figure 4).

Furthermore, we investigated whether rT1 can induce immediate skin reactions in Bet v 1-allergic patients. Three Bet v 1-allergic patients and, for control purposes, a grass pollen-allergic patient without Bet v 1-specific IgE antibodies and a non-atopic individual, were skin-prick tested with increasing concentrations of rBet v 1 and rT1, with birch pollen extract, histamine and double-distilled water. Results obtained showed that rT1 did not induce any skin reactions (wheal area) up to a concentration of 125 μg/ml (Table 2). In contrast, rBet v 1 induced immediate-type skin reactions at a concentration of 0.2 μg/ml in all three birch pollen allergic patients (Table 2). The highest concentration of 125 μg/ml rBet v 1 was not applied to patients A–C in Table 2. Birch pollen extract induced immediate skin reactions in all three birch pollen-allergic patients. Histamine induced wheal and flare reactions in all five individuals and the protein diluent (double-distilled water) failed to induce any skin reactions (Table 2).

Rabbit anti-T1 antibodies react with natural T1 and rT1, but not with most of the Bet v 1-related allergens

Using a rabbit anti-T1 antiserum raised against purified rT1, the protein could be localized by immunogold electron microscopy on ultrathin sections prepared from periwinkle callus cells by immunogold electron microscopy. Anti-T1 immunoreactivity was found to be associated with the amyloplasts (Figure 5A), whereas in control experiments performed with pre-immune antibodies almost no labelling was observed (Figure 5B).

The rabbit anti-T1 antiserum reacted with nitrocellulose-blotted rT1, as well as natural T1, and with the major carrot allergen Dau c 1. However, no reactivity with rBet v 1 and Bet v 1-related allergens (celery rApi g 1; apple rMal d 1; hazel pollen rCor a 1) was observed (Figure 5C). The pre-immune serum showed no reactivity to any of the blotted proteins (Figure 5D).

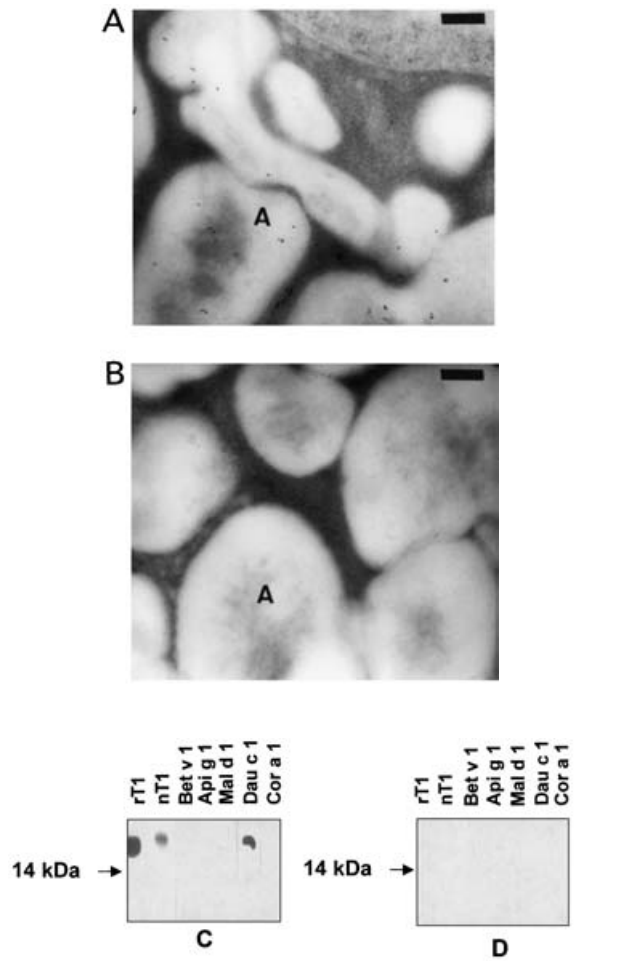

Figure 5 Localization and recognition of natural T1 and rT1 using immunogold transmission electron microscopy

(**A** and **B**) Ultrathin sections of periwinkle callus cells were probed with the rabbit anti-rT1 antiserum (**A**) and the pre-immune serum (**B**). Bound rabbit antibodies were detected with a gold conjugated goat anti-rabbit antiserum. A, amyloplast; scale bar, 0.25 μm. (**C** and **D**) Nitrocellulose-blotted rT1, natural T1 (nT1), and recombinant allergens (Bet v 1, Api g 1, Mal d 1, Dau c 1 and Cor a 1) were exposed to rabbit anti-rT1 antibodies (**C**) or to the corresponding pre-immune antibodies (**D**).

DISCUSSION

In the present study we have identified the periwinkle protein, T1, as a non-allergenic plant protein. T1 belongs to a family of proteins which were either discovered as being induced in the course of pathogen attack and, hence, were termed pathogenesis-related plant proteins (PR proteins) [1,2] or were isolated as potent allergens using IgE antibodies from allergic patients [2,4–6]. rT1 showed comparable sequence identity to the major birch pollen allergen Bet v 1 and to Bet v 1-related allergens occuring in a variety of plant species and tissues [1]. Members of the Bet v 1 allergen family represent important cross-reactive allergens in tree pollen and plant food (fruits, vegetables and spices) and thus are responsible for allergic symptoms in more than 100 million patients worldwide [2–8]. In the present study, we demonstrated that rT1 exhibits significant sequence identity with the Bet v 1 allergen family. Two regions with a length of at least nine amino acids and with a sequence identity of at least 54% among rT1, Bet v 1 and Bet v 1-cross-reactive allergens were identified. They comprised a portion of the first α-helix in Bet v 1 and the glycine-rich P-loop motif. Despite the significant sequence similarity of T1 with Bet v 1, we found that rT1 lacked allergenic potential. Although T1 contains two potential glycosylation sites which are different to those found in Bet v 1 and Bet v 1-cross-reactive allergens, it seems unlikely that post-translational modifications are responsible for the lack of allergenic activity of rT1 for at least

three reasons. Firstly, it has been found that post-translational modifications are not important for the allergenic potential of Bet v 1, because immunological equivalence between *E. coli*-expressed rBet v 1 and natural Bet v 1 has been demonstrated [34]. Secondly, we have already previously shown that natural T1 lacks IgE-binding capacity [1]. Third, none of the three Bet v 1-cross-reactive plant food allergens (apple Mal d 1; celery Api g 1; carrot Dau c 1) contains any potential glycosylation site.

It is therefore suggested to consider T1 as a substitute for Bet v 1-related allergens to construct viable transgenic plants, which neither release allergenic proteins via their pollen nor accumulate allergens in tissues used for the production of plant food. With the increasing application of molecular biology for the production of genetically engineered plants, serious concerns have been raised that genetically modified plants may represent a more potent allergen source than conventional wild-type plants [35,36]. These concerns have gained support by reports on the insertion of known plant allergens into unrelated plant species used for food production [37]. It is also becoming evident that the allergenic potential of genetically modified plants cannot be predicted by the sole analysis of the inserted gene product through the application of the principle of substantial equivalence [38]. Therefore, it has been suggested that the analysis of the allergenic potential of a given protein requires at least two different types of experiments [39]. Firstly, it has to be demonstrated that the protein is not recognized by IgE antibodies of allergic patients and does not exhibit allergenic activity in sensitized patients. Secondly, it needs to be shown that immunization with the protein in question does not induce antibodies which cross-react with known allergens to exclude sensitization *de novo* of not-yet-sensitized individuals.

To investigate whether the two requirements are fulfilled for T1, we expressed recombinant T1 in *E. coli* and purified the recombinant protein. Immunization of a rabbit with rT1 gave rise to antibodies which reacted with natural T1 suggesting immunological similarity between rT1 and natural T1. CD analysis of rT1 showed that the protein was folded, but despite the high sequence similarity of T1 with Bet v 1 and Bet v 1-related allergens, rT1 failed to react with IgE antibodies of Bet v 1-allergic patients. Furthermore, it did not induce histamine release from basophils of Bet v 1-allergic patients up to a concentration of 10 μg/ml, and caused no immediate-type skin reactions up to a concentration of 125 μg/ml. These findings demonstrate that rT1 exhibits no allergenic activity *in vitro* or *in vivo*. The most likely explanation for the divergent allergenicity of rT1 and rBet v 1 is a difference in their structure. In accordance with this assumption, the CD experiments have shown significant differences regarding secondary structure contents and thermal denaturation behaviour of rT1 and rBet v 1. Considering that Bet v 1 IgE epitopes belong mainly to the conformational type [40], it is therefore very likely that these structural differences are responsible for the different immunological features of rT1 and rBet v 1. The immunological comparison and CD analysis was only performed for the recombinant versions rT1 and rBet v 1. However, we think that these results are also valid for the natural versions of the two proteins because immunological equivalence between rBet v 1 and natural Bet v 1 has been demonstrated [34], and the fact that antibodies raised against rT1 recognized natural T1 suggests that rT1 and natural T1 also have similar immunological properties. It may therefore be assumed that natural contact with T1 or therapeutic administration of T1 will not cause allergic reactions in Bet v 1-sensitized patients.

Secondly, we found that rT1, even when administered with a strong adjuvant, failed to induce antibodies which cross-reacted with Bet v 1 or Bet v 1-related allergens, with the exception of the major carrot allergen recombinant Dau c 1. The latter finding indicates that T1 will not induce sensitization *de novo* to Bet v 1-related allergens in not-yet-sensitized individuals. Based on these findings, it is suggested to consider T1 as a paradigmatic molecule which when compared in detail by NMR or X-ray crystallography with the highly allergenic Bet v 1 molecule may teach us about the structural requirements for allergenicity of Bet v 1 and related allergens.

Furthermore, T1 may be considered as a non-allergenic substitute for Bet v 1 and Bet v 1-related allergens in genetically modified plants. Support for the feasibility of such an approach comes from the demonstration that inactivation of major allergens in rice and grass yielded plants with reduced allergenic potential [41–43]. It is suggested to continue these attempts by producing hypo-allergenic plants, where genes coding for highly allergenic molecules are replaced by genes coding for structural homologues with reduced allergenic potential. Our results, that rT1 did not give rise to antibodies which cross-reacted with Bet v 1 and most Bet v 1-related allergens, suggests that plants containing T1 instead of Bet v 1-related allergens will have a lower allergenic potential. Such transgenic plants may induce fewer allergenic responses in already sensitized patients and allow the primary prevention of allergic diseases.

In conclusion, rT1 represents a non-allergenic member of the Bet v 1 allergen family which may be useful for the construction of hypo-allergenic plants and to investigate the structural basis of Bet v 1-induced allergies.

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