

Immunological and Biological Properties of Bet v 4, a Novel Birch Pollen Allergen with Two EF-hand Calcium-binding Domains*

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We have isolated a cDNA clone coding for a birch pollen allergen, Bet v 4. The deduced amino acid sequence of Bet v 4 contained two typical EF-hand calcium-binding domains. Sequence similarities of Bet v 4 to calmodulin are primarily confined to the calcium-binding domains. However, significant sequence similarities extending outside the Ca^{2+} -binding sites were found with a recently described group of pollen-specific allergens of *Brassica* and Bermuda grass. Both EF-hand domains of Bet v 4 are able to bind Ca^{2+} , as demonstrated by $^{45}\text{Ca}^{2+}$ blot overlay of wild type and calcium-binding deficient mutants of Bet v 4. Among pollen-allergic patients, protein-bound Ca^{2+} was not an absolute requirement for IgE recognition of Bet v 4. However, disruption of the carboxyl-terminal Ca^{2+} -binding domain indicated that most IgE antibodies from allergic patients are directed against this site. IgE inhibition experiments suggested that Bet v 4 represents a highly cross-reactive pollen allergen. Pre-absorption of allergic sera with Bet v 4 drastically reduced IgE binding to proteins of similar molecular weight in pollen extracts from distantly related plant species (e.g. timothy grass, mugwort, lily) but not in extracts from plant-derived foodstuff. To test for a possible biological role in pollen germination and tube growth, we introduced recombinant Bet v 4 protein into growing lily pollen tubes by iontophoresis. As a result, cytoplasmic streaming stopped in the vicinity of the electrode tip, and a slight depolarization of the membrane voltage was measured. These effects were not observed with Ca^{2+} -binding deficient mutants of Bet v 4. Thus, Bet v 4 and homologous proteins represent a new class of pollen-specific Ca^{2+} -binding allergens that may have a physiological role as inhibitors of cytoplasmic streaming in outgrowing pollen tubes.

Atopic allergies, which comprise a wide range of IgE-mediated disorders such as hay fever, asthma, atopic dermatitis, and food allergies, affect 12–20% of the population. Pollen originating from trees, grasses, and weeds are among the most important sources of airborne allergens and are associated with seasonal patterns of allergic diseases. In addition, the patterns of sensitization of the human population are precisely related to the geographic distribution of the plants. Thus, during springtime in the temperate climate zone of the northern hemisphere, pollen grains from trees of the order Fagales (birch, hazel, alder, hornbeam, and oak) become the main source of allergens (1).

Aqueous extracts of birch (*Betula verrucosa*) pollen and related species contain a complex mixture of proteins. Approximately 40 distinct proteins are easily released from the pollen grains upon hydration, but several studies showed that only some of these proteins (approximately 10–12) are recognized by IgE from allergic individuals (2, 3). Presently, cDNA clones coding for three of these birch pollen allergens have been isolated and characterized, all showing sequence similarities to known proteins as follows: Bet v 1, the major birch pollen allergen showed significant sequence similarity to a group of plant pathogenesis-related proteins (4); Bet v 2 was identified as the actin-binding protein profilin (5); and Bet v 3 was found to belong to a novel class of calcium-binding proteins (6).

In addition to homology to pathogenesis-related proteins, Bet v 1 shows high sequence similarity to the major pollen allergens from alder (*Alnus glutinosa*) Aln g 1 (7), from hornbeam (*Carpinus betulus*) Car b 1 (8), and from hazel (*Corylus avellana*) Cor a 1 (9). This is in agreement with the clinical observation that patients displaying specific IgE to Bet v 1 also show symptoms during the flowering season of hazel and other trees belonging to the order Fagales (10). Similarly, profilin (Bet v 2) was shown to be an allergen in pollens of trees, grasses, and weeds (5), which helped to explain why some patients suffer from allergic symptoms throughout the pollen season. Bet v 1 and Bet v 2 homologous proteins were also detected in various plant-derived foodstuffs (11–13) providing an explanation for the clinical observation that birch pollen-allergic patients frequently display an intolerance to a variety of fruits and vegetables (14–16). Similar cross-reactivities were described for other allergen sources like grass pollen, mites, and animal dander. For example, albumin seems to be a common allergen responsible for cross-reactivity in dog and cat allergies as well as allergies to other mammalian allergen sources (17).

Thus, despite the relatively large number of allergens, a picture is now emerging that outlines extensive IgE cross-reactivities between homologous allergens that are present in various (sometimes unrelated) allergen sources. Based on these

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) X87153 and S54819.

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studies, it had been proposed that a reasonable number of recombinant allergens bearing most IgE epitopes can be established to be used for diagnosis and therapy of atopic allergies (18). A major advantage of this approach would be that large amounts of pure, well characterized molecules would be available permitting the precise definition of the allergen profile (allergogram) for each patient. Based on the individual IgE reactivity profile, a panel of allergens can then be selected for allergen-specific immunotherapy. Taking all this into consideration, the characterization of allergens with respect to their occurrence and IgE binding properties might help in the development of adequate tools for diagnosis and therapy of type I allergic diseases.

In this paper we describe the isolation of a cDNA coding for a novel birch pollen allergen that we designated Bet v 4. The deduced amino acid sequence of Bet v 4 showed sequence similarity to calcium-binding proteins like troponin C and calmodulin, but the similarity is basically confined to the region of the EF-hand calcium-binding domain of these proteins. Using purified recombinant Bet v 4 in IgE inhibition experiments, we could demonstrate that Bet v 4 represents a cross-reactive component in tree, grass, and weed pollen allergies. We have also performed experiments to evaluate the influence of protein-bound calcium on IgE binding properties of Bet v 4. In addition, experiments were carried out to evaluate a possible biological function of Bet v 4 in the control of calcium metabolism during pollen germination and pollen tube growth.

EXPERIMENTAL PROCEDURES

Patients and Sera—Patients allergic to birch pollen, timothy grass pollen, and mugwort pollen were selected according to typical case history, positive skin prick test, and radioallergosorbent test classes >3.5. A serum pool from non-allergic (case history, skin prick test, radioallergosorbent test) healthy donors (NHS)¹ was used as control. Sera were stored at -20 °C.

Construction and IgE Immunoscreening of a Birch Pollen cDNA Library—Total RNA was isolated from mature birch (*B. verrucosa*) pollen (Allergon AB, Engelholm, Sweden) as previously described (19) with minor modifications. Poly(A)⁺ was selected using oligo(dT)-magnetizable particles (Serotec, Oxford, UK). Superscript II reverse transcriptase (Life Technologies, Inc.) was used with 3 µg of poly(A)⁺ mRNA for the first strand cDNA synthesis. DNA polymerase I (Boehringer Mannheim, Germany) and RNase H were employed for the second strand synthesis (20). The double-stranded cDNAs were then cloned into the lambda ZAP II vector (Stratagene, La Jolla) according to the manufacturer's instructions. After *in vitro* packaging with the Gigapack Cloning Kit (Stratagene), the library was amplified once on *Escherichia coli* strain XL-1 blue. The amplified library was screened with serum IgE from a birch pollen-allergic patient. Bound IgE was detected using ¹²⁵I-rabbit anti-human IgE (Pharmacia Biotech Inc.). Isolated immunopositive phage clones were treated according to the *in vivo* excision protocol for subcloning into pBluescript SK⁺ (Stratagene). cDNA inserts were sequenced according to the dideoxy chain termination method (21).

RNA Gel Blots—Northern blot hybridization was performed using standard procedures (22). Poly(A)⁺-enriched RNA from different plant tissues was separated on a formaldehyde-containing agarose gel, blotted onto nylon membrane (Stratagene), and cross-linked to the membrane by UV irradiation. Gel-purified Bet v 4 cDNA was ³²P-labeled according to Ref. 23. The membrane was washed at 55 °C in 5 × SSPE (SSPE, 0.18 M NaCl, 10 mM Na₂HPO₄, 1 mM EDTA, pH 7.4), 0.1% SDS and then used for autoradiography.

Cloning of Bet v 4 as Non-fusion Protein in pMW175; Expression and Purification of Recombinant Bet v 4 (rBet v 4)—Expression plasmid containing the Bet v 4 cDNA was constructed in the vector pMW175 (24) which is based on the original pET vectors (25). The complete coding sequence of Bet v 4 was modified by adding an *Nco*I site at the

5' end and an *Eco*RI site at the 3' end by PCR. The following primers were used: 5'GAGACCATGGCTGATGATCATCCA3' (*Nco*I primer, *Nco*I site underlined), 5'GAGAGAATTCCTTAAATATCTTGGCAACATC3' (*Eco*RI primer, *Eco*RI site is underlined).

The PCR product was digested with *Nco*I and *Eco*RI, ligated to the respective sites of the pMW175 expression vector, and sequenced according to Ref. 21.

For expression of the pMW175/Bet v 4 plasmid, competent *E. coli* strain BL21(DE3) was transformed and selected on plates containing 100 mg/liter ampicillin (25). A single transformant colony was picked and grown to an A₆₀₀ of 1.0. Isopropyl-β-D-thiogalactopyranoside was then added to a final concentration of 1.0 mM, and incubation was continued for 6 h at 37 °C. After expression, cells were harvested by centrifugation, and pellets were resuspended in 50 mM Tris-HCl, pH 7.5, containing 220 mM NaCl. Cells were then disrupted by freezing in liquid nitrogen followed by thawing at 37 °C. This step was repeated twice. rBet v 4 was recovered in the supernatant after centrifugation at 30,000 × g for 25 min at 4 °C, which was then used for immunoblot analysis.

rBet v 4 was purified from crude *E. coli* lysates by chromatofocusing on a PBE-94 (Pharmacia) exchanger column and reversed-phase high performance liquid chromatography, as described in detail previously (26, 27).

Site-directed Mutagenesis of the EF-hand Calcium-binding Domain; Cloning, Expression, and Purification of Bet v 4 Mutants—Mutations in the two calcium-binding domains of Bet v 4 were engineered using the three-primer PCR mutagenesis method (28) to convert Asp to Ala at the first coordinating position (position x, see Fig. 1) in each EF-hand site. These Asp → Ala mutants were designated by the number of the mutated residue in the Bet v 4 amino acid sequence as follows: D19A, mutation in EF-hand site I; D54A, mutation in EF-hand site II; D19A,D54A, mutations in both EF-hand sites. Internal mismatch primers and flanking primers that were used in the present study to generate Bet v 4 mutants are listed as follows. Internal mutagenic primers: EF1 primer 5'CCATTGGCGCAAAGCGCT3'; EF2 primer 5'CCGAGATTGcCACCgATGG3'. Bases exchanged are indicated in lowercase. Flanking primers: *Nco* primer 5'GAGACCATGGCTGATGATCATCCA-3', *Nco*I site is underlined; *Eco* primer 5'GAGAGAATTCCTTAAATATCTTGGCAACATC3', *Eco*RI site is underlined.

The PCR products were digested with *Nco*I and *Eco*RI and subcloned in the pMW175 expression vector. The resulting plasmids were used to transform competent *E. coli* BL21 cells.

All PCR-amplified products were sequenced according to the dideoxy chain termination method (21). Expression in *E. coli* and purification of Bet v 4 mutants from crude bacterial lysates was done following the protocol described above for wild type rBet v 4.

MALDI-TOF MS—For mass spectrometry analysis, 0.5 µl (approximately 0.5 µg) of purified rBet v 4 and Bet v 4 mutants, 0.5 µl of protein calibration standard (insulin, average molecular weight 16,950.9), and 0.5 µl of a saturated solution of gentisic acid in 0.1% trifluoroacetic acid (matrix) were applied to the target slide with intermittent drying in an air stream. Samples were analyzed with the Kompact MALDI III mass spectrometer from Kratos Analytical in the linear flight mode. The molecular peaks of insulin and gentisic acid were used as internal standards.

Protein Extracts—Aqueous protein extracts from birch (*B. verrucosa*) pollen (Allergon AB), timothy grass (*Phleum pratense*) pollen (Allergon AB), and mugwort (*Artemisia vulgaris*) pollen (Allergon AB) were prepared by resuspending the grains in distilled water and shaking for 15 min at room temperature. Pollen grains from lily (*Lilium longiflorum*) were collected from fully developed flowers purchased from local flower shops. After washing once with 5% ethanol to remove the pollenkitt, lily pollen grains were resuspended in distilled water and shaken at room temperature for 15 min. After centrifugation, the supernatants were used for SDS-PAGE and immunoblot analysis.

SDS-PAGE, Immunoblots, IgE Binding, and IgE Inhibition Assays—Pollen extracts and bacterial lysates of rBet v 4 were analyzed by SDS-PAGE according to Laemmli (29), using 15% acrylamide gels. Proteins were visualized by staining with Coomassie Brilliant Blue R-250. For immunoblot analysis, proteins were separated by 15% SDS-PAGE and electroblotted (30) onto nitrocellulose membranes. IgE immunoblots were performed as described previously (3). Bound human IgE was detected using ¹²⁵I-rabbit anti-human IgE (Pharmacia, Uppsala, Sweden). To determine the influence of protein-bound Ca²⁺ on patients' IgE recognition of Bet v 4, during antibody binding assays nitrocellulose strips were incubated either in the presence of 0.1 mM CaCl₂ or 1 mM EGTA, pH 7.5, as described previously (6).

For IgE inhibition experiments, sera from allergic patients were

¹ The abbreviations used are: NHS, normal human serum; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; rBet v 4, recombinant Bet v 4; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; bp, base pair; Bicine, N,N-bis(2-hydroxyethyl)glycine.

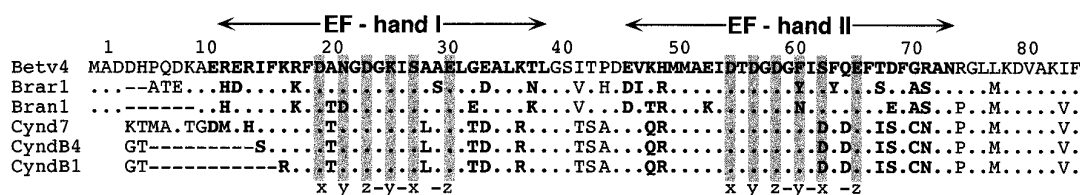


FIG. 1. Sequence comparison of Bet v 4 with related pollen allergens. The following sequences were obtained from the EMBL data base (accession numbers given in parentheses): *Brar1*, *B. rapa* pollen allergen (D63154); *Bran1*, *B. napus* pollen allergen (D63151); *Cynd7*, *Cynodon dactylon* pollen allergen (U35683); *CyndB4*, *C. dactylon* pollen allergen (A28050); *CyndB1*, *C. dactylon* pollen allergen (A28046). The two EF-hand motifs are indicated with a solid line, and residues are shown in bold characters. The coordinating residues in the calcium-binding loop are marked (x, y, z, -y, -x, -z).

preincubated with purified rBet v 4 (0.1 μ g/ml 1:10 diluted serum) overnight at 4 °C. Thereafter, blotted pollen extracts from different sources were incubated with pre-absorbed serum samples, and IgE was detected as above.

Ca²⁺-dependent Mobility Shift—Mobility shift assays were performed as described previously (31). Samples of *E. coli* lysate of recombinant Bet v 4 were adjusted to 10 mM CaCl₂ plus 2 mM EGTA, 10 mM EGTA, or 10 mM MgCl₂ plus 2 mM EGTA. 2 mM EGTA was added to all samples to chelate trace amounts of Ca²⁺ and Mg²⁺ in the protein extracts. Proteins were separated by 15% SDS-PAGE and transferred to nitrocellulose membrane. The blot was then developed using serum IgE from a birch pollen-allergic patient.

⁴⁵Ca²⁺ Blot Overlays—Purified rBet v 4 and Bet v 4 mutants were run on 15% SDS-PAGE, blotted onto polyvinylidene difluoride membranes (Millipore), and incubated with ⁴⁵Ca²⁺ (Amersham Corp., UK) in 60 mM KCl, 5 mM MgCl₂, and 10 mM imidazole, pH 6.8, for 10 min at room temperature (32). The overlay was performed using 0.1 mCi of ⁴⁵Ca²⁺/blot, corresponding to a final Ca²⁺ concentration of 64 μ M. Unbound ⁴⁵Ca²⁺ was removed by washing and bound radioactivity was visualized by exposing an x-ray film.

Cross-linking Experiments—Bet v 4 protein samples in 0.2 M Bicine, pH 8.5, were treated with 1 mM dimethyl suberimidate at 20 °C for 1 h in the presence of either 1 mM EDTA or 1 mM CaCl₂ (33). Afterward the reaction was quenched by the addition of glycine to a final concentration of 10 mM, and reaction products were analyzed by SDS-PAGE.

Circular Dichroism—CD spectra of aqueous allergen solutions were recorded as described previously (34). Briefly, cuvettes with a path length of 0.1 cm were used for collecting the sample CD spectra on a Jasco J-710 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) with a response time of 0.25 s and with a data point resolution of 0.2 nm. Each spectrum represents an average of five scans.

In Vitro Pollen Germination and Pollen Tube Culture—Dehydrated pollen grains from lily (*L. longiflorum*) were resuspended in pollen culture medium (10% (w/v) sucrose, 1 mM KCl, 0.1 mM CaCl₂, 1.6 mM H₃BO₃, pH 5.6) and incubated at room temperature. Pollen germinated after approximately 45 min, and after 2 h pollen tubes of 200–600 μ m length were collected. Pollen tube suspension (40 μ l) was pipetted onto a microscope slide and quickly but gently mixed with 60 μ l of culture medium (40 °C) additionally containing low melting agarose (Sigma, type VII) to give a final agarose concentration of 2%. Slides with immobilized pollen tubes were mounted on the stage of an inverted microscope (Nikon) equipped with a video camera to monitor cytoplasmic streaming and tube growth. In some experiments, purified rBet v 4 was added to the pollen culture medium at a concentration of 10 μ g/ml.

Electrical Measurements—The pollen tubes were impaled with microelectrodes fabricated from borosilicate glass capillaries (Clark Electromedical Instruments GC 120F) and filled with 100 mM potassium acetate, pH 7.4, as described previously (35). Microelectrodes were mounted into half cells filled with 1 M KCl. To minimize liquid junction potentials the reference electrodes were built like the microelectrodes and filled with the same solutions. Both electrodes were connected to an amplifier (Wye Science), and membrane voltage was recorded on a chart recorder. The very tip of the microelectrode was filled with 10 mM K⁺ acetate, pH 7.4, plus the recombinant wild type or mutant Bet v 4 proteins (1.8 mg ml⁻¹). The proteins were allowed to diffuse into the cytoplasm or were injected by iontophoresis using three current pulses of -2 nA and 20 s duration.

RESULTS

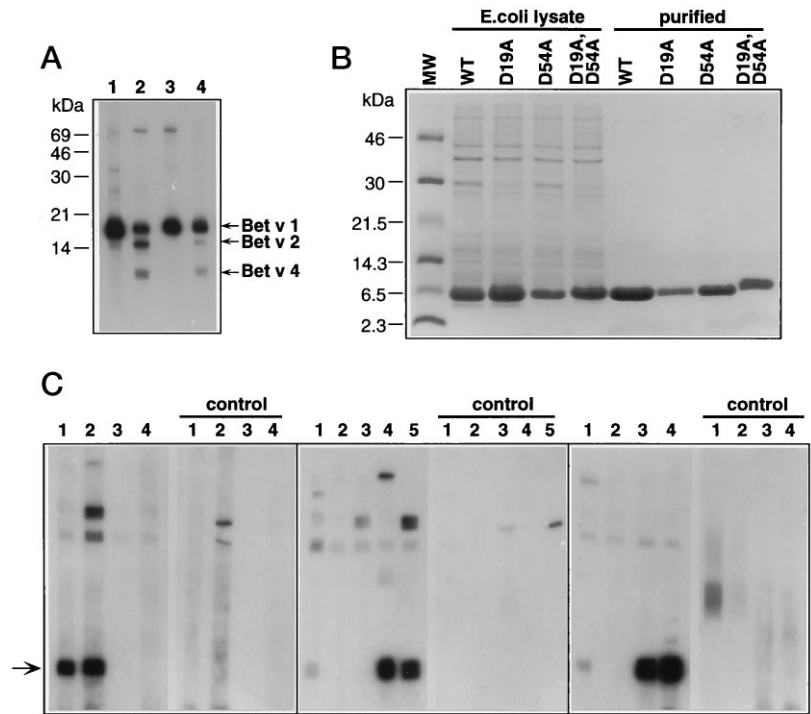
Cloning and Sequence Analysis of Bet v 4—Fig. 2A shows typical IgE binding patterns of four birch pollen-allergic patients tested on birch pollen extract. All patients reacted with

Bet v 1, the major birch pollen allergen (4), two patients recognized the ubiquitous allergen profilin (5), and patients 2 and 4 showed IgE reactivity with a so far unknown low molecular mass (approximately 7–8 kDa) protein. To obtain information about this low molecular mass allergen, serum from patient 4 was selected for IgE immunoscreening of a birch pollen cDNA expression library. Fifteen IgE-positive plaques were isolated out of 500,000 plaques and further tested for reactivity with BIP1, a monoclonal antibody raised against Bet v 1 (36) and with a rabbit serum raised against birch profilin (37). Four IgE-positive clones (c6, c7, c8, and c13) that did not react with antibodies against Bet v 1 and profilin were selected for DNA sequence analysis. All four clones were complete, and their coding regions were of identical length (252 nucleotides, excluding start and stop codons), thus coding for proteins of 84 amino acids. The sequences of c7, c8, and c13 clones were each coding for the same protein, whereas c6 differed from the other clones through two nucleotide exchanges, one that leads to amino acid exchange (Gln-6 $\rightarrow$ Arg). The 3'-noncoding regions of the four clones differed in their length (c6, 258 bp; c7, 248 bp; c8, 157 bp; and c13, 360 bp) but had identical sequences in their overlapping regions. We designated the protein encoded by clones c7, c8, and c13 as Bet v 4, in accordance with the IUIS allergen nomenclature system (38). The deduced amino acid sequence of Bet v 4 is shown in Fig. 1.

When Bet v 4 was compared with sequences in data bases, similarities were found to Ca²⁺-binding proteins such as calmodulin and troponin C from various sources and to Bet v 3, a calcium-binding allergen from birch pollen (6). However, sequence similarities between Bet v 4 and these calcium-binding proteins were primarily confined to the calcium-binding domains. Bet v 4 contained two helix-loop-helix Ca²⁺-binding regions referred to as EF-hand structures (39). The highest sequence similarities were found to a group of calcium-binding pollen allergens recently described in *Brassica* (40) and Bermuda grass (27). In this case the sequence similarities extended outside the calcium-binding domains. Fig. 1 shows the deduced amino acid sequence of Bet v 4 in its best fit alignment with the amino acid sequences of *Brassica* and Bermuda grass pollen allergens. Comparison of the amino acid sequences of *Brassica* and Bermuda grass allergens with that of Bet v 4 revealed the following identities: Bra r 1, 72%; Bra n 1, 67%; Cyn d 7, 65%; Cyn d B1, 63%; Cyn d B4, 62%. Thus, Bet v 4 and homologous proteins from *Brassica* and Bermuda grass seem to constitute a novel class of EF-hand calcium-binding proteins different from calmodulins and calmodulin-related proteins.

Expression and Purification of Wild Type Recombinant Bet v 4 (rBet v 4) and Bet v 4 Mutants—Nonfusion forms of full-length cDNA coding for Bet v 4 and three Bet v 4 mutants obtained by site-directed mutagenesis were expressed in *E. coli*. The Bet v 4 mutants D19A, D54A, and D19A,D54A were each defective in one of the two calcium-binding sites or in both sites. High levels of expression of soluble recombinant proteins

FIG. 2. Expression in *E. coli*, purification, and IgE binding to rBet v 4. **A**, IgE reactivity patterns of four birch pollen-allergic patients to proteins of a birch pollen extract separated by SDS-PAGE and blotted onto nitrocellulose membrane. Serum IgE from patient 4 was used to screen a cDNA expression library for the isolation of the Bet v 4 cDNA. **B**, Coomassie-stained 15% SDS-PAGE of lysates of *E. coli* BL21 (DE3) host strain containing expression plasmids of wild type rBet v 4 (lane WT) and calcium-binding deficient mutants of Bet v 4 (lanes D19A, D54A, and D19A,D54A), and purified recombinant proteins. **C**, IgE immunoblot of rBet v 4. Sera from four birch pollen-allergic patients (right panel, lanes 1–4), five timothy grass pollen-allergic patients (middle panel, lanes 1–5), and four mugwort pollen-allergic patients (left panel, lanes 1–4) were tested for IgE binding to rBet v 4 expressed in *E. coli*. Lysates from non-induced bacterial cells containing the rBet v 4 expression vector were used as controls.



were obtained (Fig. 2B). Wild type rBet v 4 and Bet v 4 mutants were purified to homogeneity, as determined by SDS-PAGE and Coomassie staining (Fig. 2B). More than 20 mg of each purified protein was obtained from 500-ml cultures. The protein preparations were further analyzed by MALDI-TOF mass spectrometry. The molecular masses of wild type rBet v 4, and mutants D19A, D54A, and D19A,D54A were measured as follows (theoretical mass values are given in parenthesis): 9,333.9 (9,335.8), 9,287.5 (9,291.8), 9,290.4 (9,291.8), and 9,247.0 (9,247.8), respectively. Thus, the measured mass values agree with the expected values and are consistent with the removal of the initiating methionine.

The possibility that Bet v 4 exists as a dimer was tested by *in vitro* cross-linking experiments in the presence of either EDTA or Ca^{2+} . Under the conditions applied, no dimeric or oligomeric protein bands were visible after SDS-PAGE and silver staining (data not shown).

Bet v 4 Binds Calcium—According to the deduced amino acid sequence, Bet v 4 has the potential to bind two Ca^{2+} ions. To verify whether Bet v 4 is capable of binding calcium, we performed Ca^{2+} -dependent mobility shift assays by SDS-PAGE (Fig. 3A). Such assays have been used to test calcium binding activities of calmodulin (41) and calmodulin-related proteins (31, 42). Taking as reference the relative mobility in the presence of EGTA, the mobility of Bet v 4 increased in the presence of Ca^{2+} , and no shift was detectable in the presence of Mg^{2+} (Fig. 3A). This Ca^{2+} -dependent mobility shift indicates that Bet v 4 binds calcium.

To evaluate whether both EF-hand domains are able to bind calcium, we introduced point mutations to convert Asp to Ala at the first coordinating position in each domain. This replacement is known to disrupt calcium binding to EF-hands (43), and it has been used to characterize the functional properties of different domains in several proteins including troponin C (44, 45). Fig. 3B (left panel) shows a $^{45}\text{Ca}^{2+}$ blot overlay of purified wild type rBet v 4 and Bet v 4 mutants. The D19A and D54A mutants, each containing one intact domain clearly bound calcium, although far less (approximately 50% less) than observed with wild type rBet v 4. As expected, the double mutant D19A,D54A where both domains were disrupted showed no

calcium binding activity. Therefore, we can conclude that the two EF-hand domains in the Bet v 4 sequence constitute functional calcium-binding sites.

The possibility that the conformation of Bet v 4 is affected by calcium was investigated by circular dichroism. The CD spectrum of the apo-wild type rBet v 4 (Fig. 3C, curve 1) was characteristic for a well-structured protein, showing the typical bands for α helices at 222 and 207 nm. Upon addition of Ca^{2+} (Fig. 3C, curve 2), a significant increase in the secondary structure content of wild type rBet v 4 was observed. The single point Bet v 4 mutants, D19A and D54A, exhibited a similar increase in secondary structure upon addition of Ca^{2+} (data not shown). However, the double mutant D19A,D54A remained structurally unchanged when Ca^{2+} was added (Fig. 3D).

Northern Blot Analysis—The expression of Bet v 4 homologues was examined in mature pollen of timothy grass, mugwort, and lily, as well as in leaves of mugwort (Fig. 5A). Bet v 4 homologous transcripts were detected in pollen of timothy grass (lane T), lily (lane L), and mugwort (lane M) but not in mugwort leaves (lane ML).

IgE Binding to Recombinant Bet v 4—The ability of recombinant Bet v 4 produced in *E. coli* to bind IgE was evaluated by immunoblots using sera from pollen-allergic patients. Fig. 2C shows IgE reactivity of representative pollen-allergic patients. When testing a large panel of pollen-allergic patients (100 patients), we found that approximately 5% of birch pollen-allergic patients recognized Bet v 4. To evaluate whether Bet v 4 represents a cross-reactive pollen allergen, we also tested sera from timothy grass and mugwort pollen-allergic patients. About 10–15% of timothy grass and mugwort pollen-allergic patients displayed IgE antibodies reacting with the Bet v 4 protein. In addition, IgE cross-inhibition experiments indicate that proteins with similar molecular weight in extracts from pollen of timothy grass (Fig. 5B, middle panel), lily (Fig. 5B, left panel), and mugwort (not shown) share IgE epitopes with Bet v 4. These results suggest that Bet v 4 represents a cross-reactive allergen for pollen-allergic patients.

Influence of Bound Calcium on IgE Recognition of Bet v 4—Previous studies showed that binding of monoclonal antibodies raised against calmodulin depends on calcium (46) and

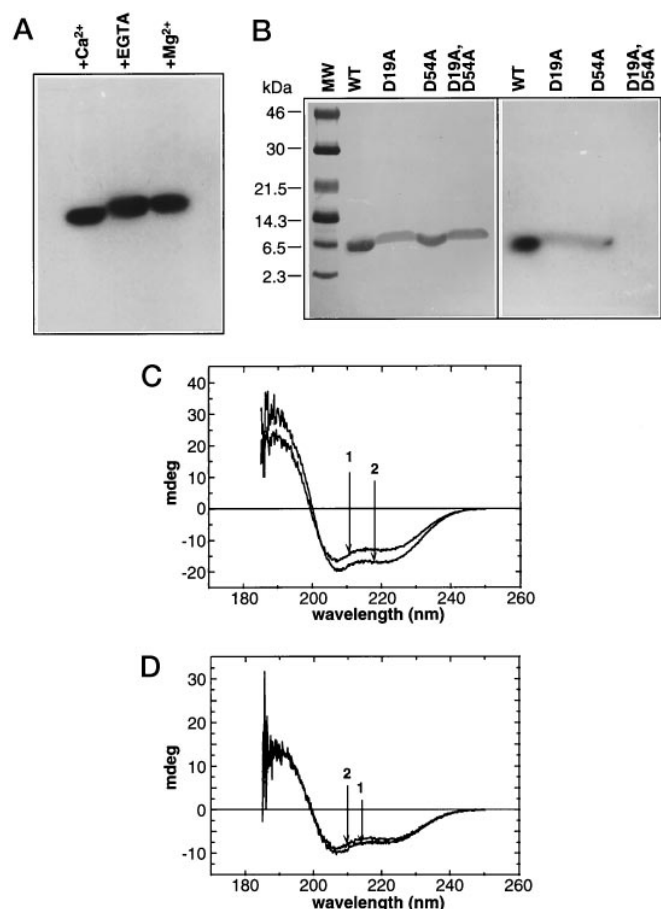


FIG. 3. Calcium binding to wild type rBet v 4 and to Bet v 4 mutants. A, bacterial lysates of wild type rBet v 4 were incubated in the presence of 10 mM CaCl_2 and 2 mM EGTA (lane Ca^{2+}), 10 mM EGTA (lane EGTA), or 10 mM MgCl_2 and 2 mM EGTA (lane Mg^{2+}), separated by SDS-PAGE, and analyzed by immunoblot with patient's serum IgE. B, Coomassie staining (right panel) and $^{45}\text{Ca}^{2+}$ blot overlay (left panel) of purified wild type rBet v 4 (lanes WT) and of calcium-binding deficient mutants of Bet v 4 (lanes D19A, D54A, and D19A,D54A). C and D, circular dichroism of wild type rBet v 4 (10 μM) and of D19A,D54A mutant (5 μM), respectively. Spectra were obtained in water (curves 1) and with 1 mM CaCl_2 (curves 2).

that IgE recognition of Bet v 3 required protein-bound Ca^{2+} (6). Consequently, we performed experiments to test the influence of calcium on IgE binding to Bet v 4. Fig. 4A shows that the effect of depletion of protein-bound Ca^{2+} using EGTA on IgE binding to rBet v 4 varied among patients. In some cases depletion of calcium did not significantly alter IgE binding to Bet v 4 (patients 2 and 3); for patients 1 and 5 a decrease in IgE binding was observed. For patient 4, IgE binding to Bet v 4 was completely abolished by depletion of protein-bound Ca^{2+} . In another set of experiments, we tested the IgE binding activities of calcium-binding deficient mutants of Bet v 4 (Fig. 4B). Disruption of the site I (D19A mutant) did alter IgE binding to Bet v 4 for patients 1 and 2, and for patient 4 a slight decrease was observed. On the other hand, loss of calcium binding activity of the site II (D54A mutant) correlated with a decrease in IgE binding to Bet v 4 for patients 1, 2, and 4. The double mutant with non-functional EF-hand sites (D19A,D54A mutant) displayed lower IgE binding activity for patients 1 and 2 and was not recognized by patient 4. The IgE reactivity patterns of individual patients with calcium-binding deficient mutants (Fig. 4B) were consistent with those observed upon depletion of protein-bound calcium (Fig. 4A).

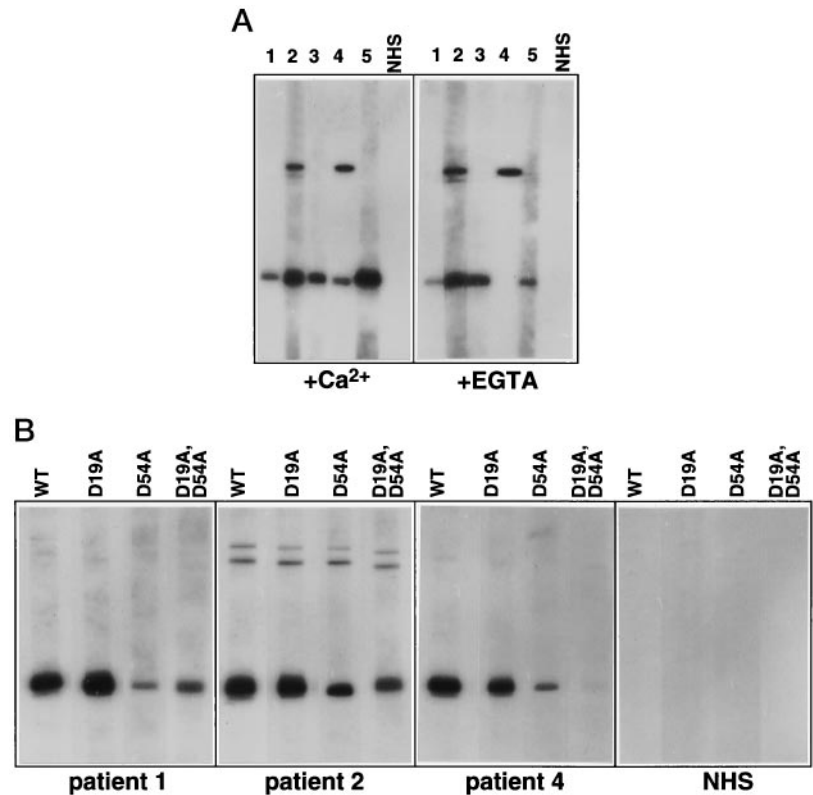
Iontophoresis of Bet v 4 into Growing Lily Pollen Tubes—To test a possible physiological role of Bet v 4, we injected the

protein into growing pollen tubes by iontophoresis and observed its effect on pollen tube growth, cytoplasmic streaming, and membrane voltage. As shown by Northern blot analysis (Fig. 5A) and IgE inhibition assays (Fig. 5B), a Bet v 4 homologous protein was identified in lily pollen. At a concentration of 1.8 mg ml^{-1} wild type rBet v 4 in the microelectrode filling, cytoplasmic streaming stopped in the vicinity of the electrode tip in approximately 30% of the experiments after an iontophoretic current pulse had been applied (Table I). In contrast, single and double mutants of the calcium-binding sites had no effect on cytoplasmic streaming when injected into growing pollen tubes. However, injected wild type rBet v 4 did not change the growth rate of pollen tubes (Fig. 6A). Control pollen tubes were growing at $7.7 \pm 2.4 \mu\text{m min}^{-1}$, whereas pollen tubes injected with rBet v 4 showed a similar growth rate of $7.5 \pm 2.6 \mu\text{m min}^{-1}$. In Fig. 6B the effect of Bet v 4 on the membrane voltage of pollen tubes is shown. A slight depolarization of the plasma membrane was observed after wild type rBet v 4 was injected into the tube's cytoplasm. Although the membrane voltage (V_m) of pollen tubes injected with rBet v 4 was not statistically different from the V_m of control tubes ($p = 0.088$, Student's t test), injection of single and double mutants of the Bet v 4 protein showed much lower (single mutant D19A, $p = 0.59$) or even no membrane depolarization effect (single mutant D54A, double mutant D19A,D54A). Control experiments with the microelectrode filling without added proteins were also performed and had no effect on tube morphology, membrane voltage, and cytoplasmic streaming.

DISCUSSION

Molecular and Immunological Characterization of Bet v 4—We have isolated a complete cDNA encoding a novel birch pollen allergen Bet v 4. This protein has 84 amino acids and a calculated molecular mass of 9335.8 Da. The deduced amino acid sequence indicates that Bet v 4 contains two potential Ca^{2+} -binding sites of the EF-hand type (47). Sequence similarities of Bet v 4 to calmodulins, to a calcium-binding allergen from birch pollen Bet v 3, and other EF-hand calcium-binding proteins are primarily confined to the calcium-binding sites. Bet v 4 is significantly shorter than Bet v 3 and calmodulins. Bet v 3 has 203 amino acids and three calcium-binding sites, and calmodulin has 149 amino acids and four calcium-binding sites. High sequence similarities extending outside the calcium-binding domains were found to Bra n 1 and Bra r 1 from *Brassica* pollen and to Cyn d 7, Cyn d B4, and Cyn d B1 from Bermuda grass pollen. These proteins were recently described as calcium-binding allergens in pollen from members of the *Brassica* family (*Brassica rapa* and *Brassica napus*) (40) and Bermuda grass (27). Similar to Bet v 4, these allergens are all small proteins (71–83 amino acids) containing two calcium-binding sites with the same structural spacing. EF-hand proteins are presently classified into 32 distinct subfamilies of proteins that contain two to eight EF-hand domains. Members of the S100 protein subfamily also have approximately the same molecular weight as Bet v 4 and two EF-hand motifs (48). However, sequence similarities between Bet v 4 and S100 proteins are evident only in the carboxyl-terminal EF-hand domain. In addition, Bet v 4 and homologous proteins have two canonical EF-hand loops of 12 amino acids, whereas the S100 proteins typically contain two distinct EF-hand calcium-binding loops of 14 and 12 amino acids, respectively. Most S100 proteins are known to exist as dimers, and both disulfide-bound and noncovalently associated forms were described (33, 49). We tested this possibility for Bet v 4 by *in vitro* cross-linking experiments, but no dimeric or oligomeric forms were detected. Therefore, we propose that Bet v 4 and homologous proteins constitute a new subfamily of EF-hand calcium-binding proteins.

FIG. 4. Influence of protein-bound calcium on IgE binding to Bet v 4. **A**, bacterial lysates containing wild type rBet v 4 were separated by SDS-PAGE, blotted onto nitrocellulose membranes, and tested for IgE reactivity using sera from five pollen-allergic patients (lanes 1–5) and a serum pool from non-allergic donors (NHS). Membranes were incubated with the addition of either 10 mM CaCl_2 ($+\text{Ca}^{2+}$) or 10 mM EGTA ($+\text{EGTA}$). **B**, IgE binding to calcium-binding deficient mutants of Bet v 4. *E. coli* lysates containing wild type rBet v 4 (lanes WT) and Bet v 4 mutants with one (lanes D19A and D54A) or both (lane D19A, D54A) calcium-binding sites disrupted were tested for IgE binding with sera from pollen-allergic patients and with a serum pool from non-allergic individuals (NHS). Sera used for the experiments in **B** were obtained from the same allergic individuals, as indicated in **A**.



A significant percentage of known EF-hand domains do not bind calcium (50). Our results demonstrated that Bet v 4 is capable of binding calcium. In addition, $^{45}\text{Ca}^{2+}$ blot overlay of wild type and of Bet v 4 mutants that are defective in the calcium-binding loops revealed that both sites contribute to the calcium binding activity of Bet v 4. Regarding specificity, EF-hand sites are either calcium-specific (Ca^{2+} sites) or capable of binding both calcium and magnesium ($\text{Ca}^{2+}/\text{Mg}^{2+}$ sites). Although the structural basis for divalent metal discrimination by EF-hand sites still remains unknown, it was found that the replacement of the aspartic acid present in the 12th position of a $\text{Ca}^{2+}/\text{Mg}^{2+}$ site with a glutamic acid increased calcium affinity and abolished magnesium binding, thus rendering the site calcium-specific (51). The Bet v 4 protein has a glutamic acid in position 12 of both metal-binding loops, and no shift in the mobility was detectable in the presence of Mg^{2+} suggesting that the Bet v 4 metal-binding sites are calcium-specific.

The importance of protein-bound calcium on antibody recognition has been documented for calmodulin and Bet v 3 (6, 46). Our experiments using EGTA to deplete protein-bound calcium indicated that for some patients IgE recognition of Bet v 4 required calcium. But in most cases, substantial IgE binding could still be detected after depletion of bound calcium. Although our CD measurements showed significant Ca^{2+} -induced structural changes of Bet v 4, apparently some of the IgE epitopes are still maintained in apo-Bet v 4. Recent progress of atomic resolution structural analysis of several EF-hand proteins has provided valuable information about the conformational change induced by calcium binding (reviewed in Ref. 52). Direct comparison of the solution (NMR) structures of apo- and Ca^{2+} -loaded forms of calmodulin showed that the secondary structure is conserved between the two forms, whereas the tertiary structure changes dramatically from a compact regular four-helix bundle in the apo form to a more extended, open structure in the Ca^{2+} -loaded form (53). The conservation of secondary structure between the two forms has led to the conclusion that the Ca^{2+} -induced structural differences for cal-

modulin, as measured by CD, are not related to a change in helical content but more likely to changes in spatial orientation of helices (53). Therefore, our CD data showing increase in secondary structure of Bet v 4 in the presence of calcium alternatively could be interpreted as changes in the tertiary structure.

Interestingly, calcium-binding deficient mutants of Bet v 4 indicated that most Bet v 4-specific IgE antibodies in birch pollen-allergic patients are directed to the carboxyl-terminal calcium-binding domain. This also seems to be true for Cyn d 7, the Bet v 4 homologue in Bermuda grass pollen. Synthetic peptides (12-mer) based on the amino acid sequences of the two Cyn d 7 calcium-binding domains were tested for their IgE binding capacity. Only the peptide corresponding to the carboxyl-terminal calcium-binding domain bound IgE (27). Furthermore, when preincubated with sera from allergic patients, this peptide significantly inhibited IgE binding to Cyn d 7 as well as to Bet v 4. The carboxyl-terminal calcium-binding domain of Bet v 4 and Cyn d 7 shows 84% sequence identity. The combined results support the notion that the carboxyl-terminal calcium-binding domain of Bet v 4 (and very likely also of homologous allergenic proteins in other plants) represents a major continuous (linear) IgE epitope, although it might also be possible that this linear domain is part of a discontinuous (conformation-dependent) epitope(s) on the folded proteins.

The fact that patients sensitized to timothy grass pollen and mugwort pollen showed IgE reactivity to rBet v 4 suggests that Bet v 4 represents a cross-reactive allergen. Indeed, the addition of purified rBet v 4 to sera from allergic patients significantly reduced IgE binding to low molecular weight proteins in pollen extracts of timothy grass, mugwort, and lily. Purified rBet v 4 also efficiently competed with Cyn d 7 for the same IgE antibodies (27). However, in IgE inhibition experiments we were not able to detect cross-reacting proteins in extracts of apple and celery, and no positive clones could be isolated by screening apple and celery cDNA libraries using the Bet v 4

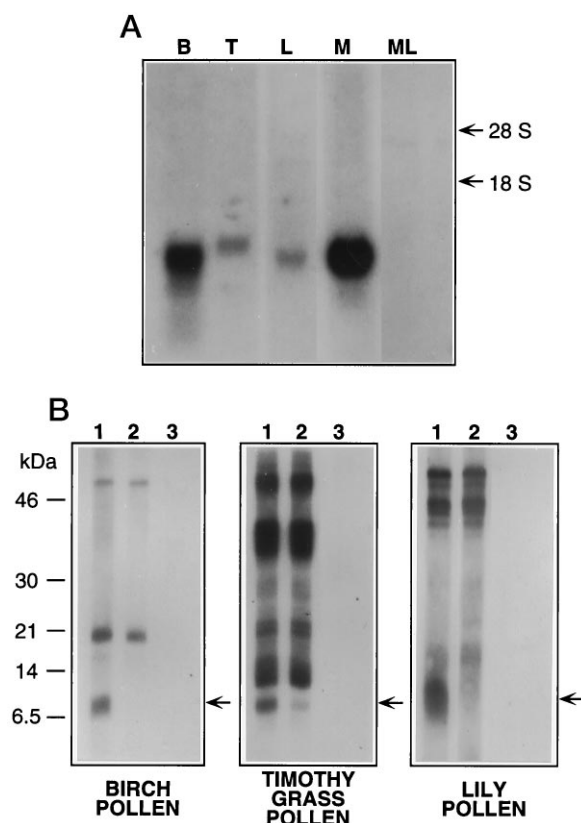


FIG. 5. **Bet v 4 homologues in pollen from different plant species.** A, Northern blot analysis of poly(A)⁺-enriched RNA isolated from mature birch (*B. verrucosa*) pollen (lane B), timothy grass (*P. pratense*) pollen (lane T), lily (*L. longiflorum*) pollen (lane L), mugwort (*A. vulgaris*) pollen (lane M), and from mugwort leaves (lane ML). B, IgE cross-inhibition experiments. Protein extracts from birch pollen, timothy grass pollen, and lily pollen were separated by SDS-PAGE, blotted onto nitrocellulose membranes, and probed with serum from a pollen-allergic patient only (lanes 1) or with serum preincubated with 0.1 µg/ml purified wild type rBet v 4 (lanes 2). Lanes 3 are controls leaving out the serum incubation step.

TABLE I

Cytoplasmic streaming in growing lily pollen tubes after iontophoresis of purified wild type rBet v 4 and calcium-binding deficient mutants of Bet v 4 (D19A, D54A and D19A,D54A)

	Cytoplasmic streaming	
	Continued	Stopped
rBet v 4 (n = 29)	20 (69%)	9 (31%)
D19A (n = 13)	13 (100%)	0 (0%)
D54A (n = 10)	10 (100%)	0 (0%)
D19A,D54A (n = 9)	9 (100%)	0 (0%)
Control (n = 73)	71 (97%)	2 (3%)

cDNA as a probe.² These findings correlate well with results obtained on the analysis of expression by Northern hybridization. Bet v 4 homologous transcripts were detectable in RNA from mature timothy grass pollen, mugwort pollen, and lily pollen but not in mugwort leaves. Cyn d 7 transcripts were detectable in mature pollen RNA but not in RNA from other

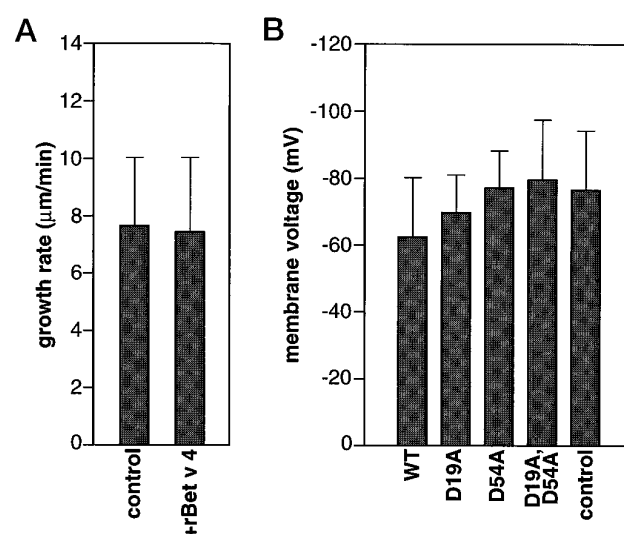


FIG. 6. **Pollen tube growth and membrane voltage measurements after iontophoresis of wild type rBet v 4 and Bet v 4 mutants into growing lily pollen tubes.** A, growth rate of lily pollen tubes impaled with microelectrodes filled at the very tip with 10 mM K⁺ acetate, pH 7.4 (control), or with 10 mM K⁺ acetate, pH 7.4, plus 1.8 mg/ml purified wild type rBet v 4 (+rBet v 4). The values are means ± S.D. (control, n = 62; +rBet v 4, n = 21). B, membrane voltage (V_m) of growing lily pollen tubes measured after injection of purified wild type rBet v 4 (WT), of calcium-binding deficient mutants of Bet v 4 (D19A, D54A, and D19A,D54A), or buffer alone without added proteins (control). The values are means ± S.D. (WT, n = 21; D19A, n = 13; D54A, n = 11; D19A, D54A, n = 9; control, n = 79).

Bermuda grass tissues. Moreover, Cyn d 7 homologous transcripts were identified in mature pollen from 13 other grasses (27). Bra r 1 transcripts were detected in anthers during pollen maturation and in mature pollen stage but not in stigmas or leaves (40). Altogether, these data indicate that proteins of the Bet v 4 family are highly cross-reactive allergens that are specifically expressed in pollen from distantly related plants but do not seem to be present in plant-derived foodstuff. It is interesting to note that *Brassica* and *Lilium* plants are insect pollinated and produce almost no airborne pollen. Therefore, allergy to *Brassica* or *Lilium* pollen is uncommon in the population. However, epidemiological studies showed that the incidence of *Brassica* pollen allergy is much higher in individuals occupationally exposed to *Brassica* pollen (54).

Possible Physiological Function of Bet v 4—It is well established that calcium ions play a critical role in pollen germination and in particular in pollen tube growth (55, 56). Growing pollen tubes show a substantial tip-focused gradient of free calcium ions in the cytoplasm with a maximum in the very tip of the tube (57, 58), whereas nongrowing tubes either do not have a gradient or do have a negative gradient (59). The observation that transcripts of Bet v 4 and Bet v 4-like proteins are present in mature pollen stage suggests that these proteins might function in the regulation of calcium metabolism during pollen germination and pollen tube growth. Unfortunately, optimal conditions for *in vitro* culture of birch pollen are not yet established. Therefore, we used in our experiments the *Lilium* system, where it is possible to induce pollen activation and germination *in vitro* by incubation in an appropriate growth medium. In our experiments, Bet v 4 and homologous proteins were rapidly released (within 15 min) when pollen grains were hydrated in water. Furthermore, addition of Bet v 4 to the germination medium did not affect the germination rate of lily pollen as well as tube growth (data not shown). However, when rBet v 4 was introduced by iontophoresis into growing lily pollen tubes, cytoplasmic streaming stopped in the vicinity of

² E. Engel, K. Richter, G. Obermeyer, P. Briza, A. J. Kungl, B. Simon, M. Auer, C. Ebner, H.-J. Rheinberger, M. Breitenbach, and F. Ferreira, unpublished observations.

the electrode tip in 30% of the cases, and a slight depolarization of the plasma membrane was measured. These effects were not observed with calcium-binding deficient mutants of Bet v 4. Taking all these aspects into consideration, we would like to propose that Bet v 4 acts as a growth inhibitor in resting pollen grains. Since in most plant species, pollen germination and tube growth are relatively rapid events, it seems reasonable to assume that pollen exudation could be an efficient mechanism for quick removal of metabolic inhibitors allowing the activation of the synthetic machinery of the cell and reorganization of the cytoskeleton, which is necessary for the polarized growth of pollen tubes. It should be pointed out that botanically *Betula* and *Lilium* (a dicot and a monocot, respectively) are not closely related. But still Bet v 4 could perturb cytoplasmic streaming in lily pollen tubes in a calcium-dependent manner. Efforts are underway in our laboratory to isolate the Bet v 4-homologue in lily pollen as a first step to further investigate its role in pollen biology.

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REFERENCES

- D'Amato, G., Spieksma, F. T. M., and Bonini, S. (1991) *Allergenic Pollen and Pollinosis in Europe*, Blackwell, Oxford
- Viander, M., Fräki, J., Djupsund, B. M., and Laine, S. (1979) *Allergy (Cph.)* **34**, 289–302
- Jarolim, E., Rumpold, H., Endler, A. T., Ebner, H., Breitenbach, M., Scheiner, O., and Kraft, D. (1989) *Allergy (Cph.)* **44**, 385–395
- Breiteneder, H., Pettenburger, K., Bito, A., Valenta, R., Kraft, D., Rumpold, H., Scheiner, O., and Breitenbach, M. (1989) *EMBO J.* **8**, 1935–1938
- Valenta, R., Duchene, M., Ebner, C., Valent, P., Sillaber, C., Deviller, P., Ferreira, F., Tejkl, M., Edelmann, H., Kraft, D., and Scheiner, O. (1992) *J. Exp. Med.* **175**, 365–374
- Seiberler, S., Scheiner, O., Kraft, D., Lonsdale, D., and Valenta, R. (1994) *EMBO J.* **13**, 3481–3486
- Breiteneder, H., Ferreira, F., Reikerstorfer, A., Duchene, M., Valenta, R., Hoffmann-Sommergruber, K., Ebner, C., Breitenbach, M., Kraft, D., and Scheiner, O. (1992) *J. Allergy Clin. Immunol.* **90**, 909–917
- Larsen, J. N., Ströman, P., and Ipsen, H. (1992) *Mol. Immunol.* **29**, 703–711
- Breiteneder, H., Ferreira, F. D., Hoffmann-Sommergruber, K., Ebner, C., Breitenbach, M., Rumpold, H., Kraft, D., and Scheiner, O. (1993) *Eur. J. Biochem.* **212**, 355–362
- Dirksen, A., and Østerballe, O. (1978) *Allergy (Cph.)* **33**, 299–309
- Ebner, C., Hirschwehr, R., Bauer, L., Breiteneder, H., Valenta, R., Ebner, H., Kraft, D., and Scheiner, O. (1995) *J. Allergy Clin. Immunol.* **95**, 962–969
- Vanek-Krebitz, M., Hoffmann-Sommergruber, K., Laimer da Camera Machado, M., Susani, M., Ebner, C., Kraft, D., and Scheiner, O. (1995) *Biochem. Biophys. Res. Commun.* **214**, 538–551
- Breiteneder, H., Hoffmann-Sommergruber, K., O'Riordain, G., Susani, M., Ahorn, H., Ebner, C., Kraft, D., and Scheiner, O. (1995) *Eur. J. Biochem.* **233**, 484–489
- Eriksson, N. E., Formgren, H., and Svenonius, E. (1982) *Allergy (Cph.)* **37**, 437–443
- Loewenstein, H., and Eriksson, N. E. (1983) *Allergy (Cph.)* **38**, 162–172
- Van Ree, E., and Aalberse, R. C. (1993) *J. Clin. Immunoassay* **16**, 124–130
- Spitzauer, S., Pandjaitan, B., Söregi, M. D., Mühl, S., Ebner, C., Kraft, D., Valenta, R., and Rumpold, H. (1995) *J. Allergy Clin. Immunol.* **96**, 951–959
- Valenta, R., and Kraft, D. (1995) *Curr. Opin. Immunol.* **7**, 751–756
- Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- Gubler, U., and Hoffman, B. J. (1983) *Gene (Amst.)* **25**, 263–269
- Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463–5467
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (eds) (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Feinberg, A. P., and Vogelstein, B. (1984) *Anal. Biochem.* **137**, 266–267
- Way, M., Pope, B., Gooch, J., Hawkins, M., and Weeds, A. G. (1990) *EMBO J.* **9**, 4103–4109
- Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) *Methods Enzymol.* **185**, 60–89
- Ferreira, F. D., Hoffmann-Sommergruber, K., Breiteneder, H., Pettenburger, K., Ebner, C., Sommergruber, W., Steiner, R., Bohle, B., Sperr, W. R., Valent, P., Kungl, A. J., Breitenbach, M., Kraft, D., and Scheiner, O. (1993) *J. Biol. Chem.* **268**, 19574–19580
- Suphioglu, C., Ferreira, F., and Knox, R. B. (1997) *FEBS Lett.* **402**, 167–172
- Marini, F., III, Naeem, A., and Lapeyre, J.-N. (1993) *Nucleic Acids Res.* **21**, 2277–2278
- Laemmli, U. K. (1970) *Nature* **227**, 680–685
- Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 4350–4354
- Sistrunk, M. L., Antosiewicz, D. M., Purugganan, M. M., and Braam, J. (1994) *Plant Cell* **6**, 1553–1565
- Pedrocchi, M., Schäfer, B. W., Durussel, I., Cox, J. A., and Heizmann, C. W. (1994) *Biochemistry* **33**, 6732–6738
- Dell'Angelica, E. C., Schleicher, C. H., and Santome, J. A. (1994) *J. Biol. Chem.* **269**, 28929–28936
- Kungl, A. J., Susani, M., Lindemann, A., Machius, M., Visser, A. J. W. G., Scheiner, O., Kraft, D., Breitenbach, M., and Auer, M. (1996) *Biochem. Biophys. Res. Commun.* **223**, 187–192
- Obermeyer, G., and Blatt, M. R. (1995) *J. Exp. Bot.* **46**, 803–813
- Jarolim, E., Tejkl, M., Rohac, M., Schlerka, G., Scheiner, O., Kraft, D., Breitenbach, M., and Rumpold, H. (1989) *Int. Arch. Allergy Appl. Immunol.* **90**, 54–60
- Valenta, R., Ferreira, F., Grote, M., Swoboda, I., Vrtala, S., Duchene, M., Deviller, P., Meagher, R. B., McKinney, E., Heberle-Bors, E., Kraft, D., and Scheiner, O. (1993) *J. Biol. Chem.* **268**, 22777–22781
- King, T. P., Hoffman, D., Loewenstein, H., Marsh, D. G., Platts-Mills, T. A. E., and Thomas, W. (1995) *J. Allergy Clin. Immunol.* **96**, 5–14
- Kretsinger, R. H., and Nockolds, C. E. (1973) *J. Biol. Chem.* **248**, 3313–3326
- Toriyama, K., Okada, T., Watanabe, M., Ide, T., Ashida, T., Xu, H., and Singh, M. B. (1995) *Plant Mol. Biol.* **29**, 1157–1165
- Burgess, W. H., Jemiolo, D. K., and Kretsinger, R. H. (1980) *Biochim. Biophys. Acta* **623**, 257–270
- Ling, V., and Zielinski, R. E. (1993) *Plant Mol. Biol.* **22**, 207–214
- Reinach, F. C., Nagai, K., and Kendrick-Jones, J. (1986) *Nature* **322**, 80–83
- Farah, C. S., Miyamoto, C. A., Ramos, C. H. L., da Silva, A. C. R., Quaggio, R. B., Fujimori, K., Smillie, L. B., and Reinach, F. C. (1994) *J. Biol. Chem.* **269**, 5230–5240
- Sorenson, M. M., da Silva, A. C. R., Gouveia, C. S., Sousa, V. P., Oshima, W., Ferro, J. A., and Reinach, F. C. (1995) *J. Biol. Chem.* **270**, 9770–9777
- Hulen, D., Baron, A., Salisbury, J., and Clarke, M. (1991) *Cell Motil. Cytoskeleton* **18**, 113–122
- Kretsinger, R. H. (1980) *CRC Crit. Rev. Biochem.* **12**, 119–174
- Schäfer, B. W., and Heizmann, C. W. (1996) *Trends Biochem. Sci.* **21**, 134–140
- Selinfreund, R. H., Barger, S. W., Pledger, W. J., and Van Eldik, L. J. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 3554–3558
- Kretsinger, R. H., Tolbert, D., Nakayama, S., and Pearson, W. (1991) *Novel Calcium Binding Proteins* (Heizmann, C., ed) pp. 17–38, Springer-Verlag, Heidelberg
- da Silva, A. C. R., Kendrick-Jones, J., and Reinach, F. C. (1995) *J. Biol. Chem.* **270**, 6773–6778
- Ikura, M. (1996) *Trends Biochem. Sci.* **21**, 14–17
- Finn, B., B. E., Evenäs, J., Drakenberg, T., Waltho, J. P. Thulin, E., and Forsen, S. (1995) *Nat. Struct. Biol.* **2**, 777–783
- Fell, P. J., Soulsby, S., Blight, M. M., and Brostoff, J. (1992) *Clin. Exp. Allergy* **22**, 501–505
- Brewbaker, J. L., and Kwack, B. H. (1963) *Am. J. Bot.* **50**, 859–865
- Steer, J. M., and Steer, M. W. (1989) *New Phytol.* **111**, 323–358
- Nobiling, R., and Reiss, H. D. (1987) *Protoplasma* **139**, 20–24
- Obermeyer, G., and Weissenfeld, M. H. (1991) *Eur. J. Cell Biol.* **56**, 319–327
- Rathore, K. S., Cork, R. J., and Robinson, K. R. (1991) *Dev. Biol.* **148**, 612–619