

Common epitopes of birch pollen and apples—Studies by Western and Northern blot

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Eighty-three sera from patients with birch-pollen allergy were investigated for IgE antibodies against apple allergens by means of immunoblotting. In immunoblots, 81 patients (97.6%) exhibited IgE directed against the major allergen of birch, Bet v I (17 kd), and these patients also demonstrated IgE binding to apple allergens in the molecular weight range 17 to 18 kd. Inhibition studies by preincubation of sera with birch-pollen extract led to complete blocking of IgE binding to this 17 to 18 kd protein, whereas preincubation with apple extract could not diminish IgE binding to Bet v I. Furthermore, a 17 kd protein in apple extract could be detected by immunoblotting with a Bet v I-specific monoclonal antibody. Northern blotting with a Bet v I cDNA clone as a probe revealed cross-hybridization of birch and apple allergen coding nucleic acids under conditions of high stringency, suggesting significant homology of the nucleic acid level. Our results support the concept that antigens in birch pollen and apples share allergenic epitopes leading to IgE cross-reactivities that may cause clinical manifestations when a special threshold level of specific IgE antibodies is reached. (J ALLERGY CLIN IMMUNOL 1991;88:588-94.)

Key words: Birch pollen, apples, IgE cross-reactivity, monoclonal antibody, cDNA Bet v I

The clinical observation that patients suffering from type I allergy caused by birch pollen frequently demonstrate an intolerance to fruits and/or vegetables has already been described in several publications.¹⁻¹¹ Eriksson et al.⁴ designated the responsible agents as BPRF. Among these nourishments, the most frequent and important are apples. Inhibition studies of IgE binding to birch pollen and apple extracts in RAST suggested the existence of IgE-binding epitopes common to birch-pollen proteins and apple proteins.^{3, 6} However, it could not be unequivocally demonstrated that the major allergen of birch pollen, *Bet v I*,^{12, 13} or one or several of the minor birch-pollen allergens

Abbreviations used

BPRF:	Birch-pollen related food stuff
GD:	Golden delicious apples
MW:	Molecular weight
NC:	Nitrocellulose
MAb:	Monoclonal antibody
cDNA:	Complementary deoxyribonucleic acid
Eco R I:	Restriction endonuclease
Hinf I:	Restriction endonuclease
SSC:	Sodium citrate buffer
RT:	Room temperature
Ab:	Antibody

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were responsible for putative cross-reactions with apple allergens. Nonspecific mechanisms, like lectin-IgE interactions, unspecific histamine release, and even contamination of the fruits with microfungi or birch pollen have been discussed.^{2, 9} The aim of the present study was to compare Ab-binding components in birch pollen and apple extracts by the use of patients' IgE and MAbs to identify common allergens. In addition, DNA/RNA technology was used to detect homologue structures at the nucleic acid level.

MATERIAL AND METHODS

Patients

Sera from 83 birch pollen-allergic patients suffering from birch-pollen allergy, proven by case history, positive skin prick test, and positive RAST, were selected for this study (34 men and 49 women; age ranging from 10 to 60 years; median, 34 years). Most of these patients, 63/83 (75.9%), demonstrated clinical symptoms (ranging from itching in the mouth or rhinitis to urticaria/angioedema) after ingestion of apples. The severity of these symptoms varied according to pollen counts during the birch-pollen season and were weaker in the pollen-free seasons.

Sera collected from 10 individuals without *in vivo* or *in vitro* indications of any type I allergy served as control group.

Birch-pollen extract

Protein extraction of birch pollen (*Betula verrucosa*) was performed as previously described.¹⁴

Apple extracts

Extraction of apple extracts was performed from GD and Granny Smith apple according to the method of Björkstén et al.¹⁵ Homogenized pieces of frozen apple were mixed with 10 mmol/L of potassium phosphate buffer (pH 7.0) containing 2% suspended solid polyvinylpyrrolidone, 2 mmol/L of ethylenediaminetetraacetic acid disodium salt, 10 mmol/L of sodium diethyldithiocarbamate, and 3 mmol/L of sodium azide. The homogenate was divided into three fractions. Fraction 1 was stirred vigorously for 10 minutes and then centrifuged at 40,000 g for 30 minutes at RT. Fractions 2 and 3 were shaken at 4° C for 2 hours and overnight, respectively, and then centrifuged at 40,000 g for 30 minutes at RT. A mixture of the three supernatants was dialyzed against 10 mmol/L of potassium phosphate buffer (pH 7.0) containing 3 mmol/L of sodium azide for 20 hours at 4° C. Thereafter, the extract was lyophilized and stored at -20° C. Since GD is the most frequent apple reported by patients to cause intolerance, this apple was used for our study.

Hazelnut, poppy seed, and banana extracts

Hazelnut, poppy seed, and banana were extracted in the same way described for apples, and extracts were used for IgE immunoblotting experiments.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and protein transfer

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as previously described.¹⁴ For our experiments, we used 15% homogeneous gels; 3 mg/ml per gel of birch-pollen extract was applied. In the case of apple extract, 5 mg/ml per gel was applied. By pilot titration experiments, these concentrations were found to be ideal for our assays. The separated proteins were transferred to NC by use of a blotting apparatus (Hoefer Scientific In-

struments, San Francisco, Calif.) at 150 mA for 6 hours at RT. This procedure guaranteed complete transfer of proteins to NC membranes.

IgE blots

IgE blots were performed in the following buffer system: 50 mmol/L of sodium phosphate, 0.5% (vol/vol) of Tween 20, 0.5% (wt/vol) of bovine serum albumin, and 0.05% (wt/vol) of sodium azide, pH 7.5. NC membranes were cut into 5 mm strips and were then saturated in buffer for 30 minutes. Thereafter, NC strips were incubated with 1 ml of patients' sera diluted 1:4 in buffer, under continuous shaking at 4° C, overnight. After a wash with buffer for 30 minutes, NC strips were incubated with ¹²⁵I-labeled rabbit antihuman IgE (Pharmacia, Uppsala, Sweden) and diluted in buffer to achieve 300,000 cpm per strip. Thereafter, NC membranes were washed and subjected to autoradiography (-70° C for 48 hours) with high-performance autoradiography films (Hyperfilm MP, Amersham International plc., Amersham, England) in exposure cassettes (X-OMATIC, Kodak-Austria, Vienna, Austria).

For inhibition experiments, sera from 32 patients exclusively reacting with the major allergen of birch, *Bet v* I,^{12, 21} were pooled. The pool was diluted 1:4 in the above-mentioned buffer, and 1 ml was incubated under continuous shaking at 4° C overnight after addition of 0, 0.0625, 0.125, 0.250, and 0.500 mg, respectively, of birch extract before application to apple extract-coated strips, and of 0, 0.5, 1, 2, and 5 mg, respectively, of apple extract before application to birch-pollen extract-coated strips.

The same pool was used in immunoblot assays with hazelnut, poppy seed, and banana.

MAB blots

The following MABs were used: BIP 1 (Jarolim et al.¹⁶), a MAB detecting an epitope of *Bet v* I, BIP 4, also reacting exclusively with *Bet v* I but obviously with a distinct epitope.¹⁷ MABs were blotted with the following buffer system: 50 mmol/L of Tris, 150 mmol/L of NaCl, and 0.5% (vol/vol) of Tween 20, pH 7.4. First, NC strips were saturated in buffer supplemented with 3% powdered milk. Subsequently, NC strips were incubated with concentrated hybridoma supernatants under continuous shaking, at 4° C, overnight. After a wash with the above-mentioned buffer for 30 minutes, strips were incubated with ¹²⁵I-labeled sheep antimouse immunoglobulin (Amersham International plc.), diluted 1:1000 in buffer (300,000 cpm/ml), and supplemented with 3% powdered milk for 1 hour at RT. After a new washing procedure, NC membranes were subjected to autoradiography as described above.

RNA extraction and Northern blotting

RNA extraction from apple (GD, 200 gm) was performed as described for male inflorescences and pollen from birch¹⁸; 30 µg of total apple RNA was loaded onto each lane of a gel and separated through 1.1% agarose gel containing 6%

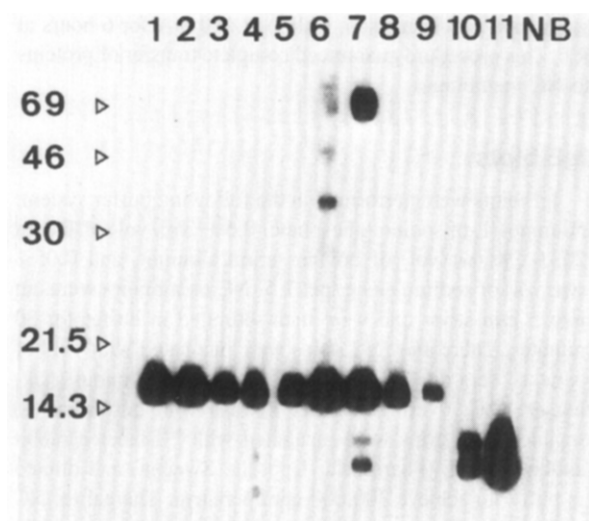


FIG. 1. IgE immunoblots with sera from 11 selected patients on birch-pollen extract-coated NC strips; *patients 1 to 5 and 8 and 9*: IgE binding to the major allergen, *Bet v I*; *patients 6 and 7*: IgE binding to *Bet v I* and to minor allergens; *patients 10 and 11*: patients reacting with a minor allergen in MW range of 13 kd, exclusively.

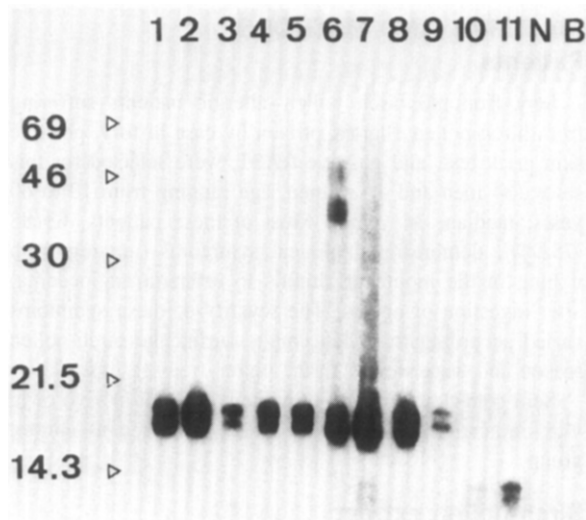


FIG. 2. IgE immunoblots with sera from 11 selected patients on apple extract-coated NC strips; *patients 1 to 5 and 8 and 9*: IgE binding in the MW range 17 to 18 kd; *patients 6 and 7*: IgE binding to the major allergen and to minor allergens; *patients 10 and 11*: patients reacting with a minor allergen in the region 13 kd, exclusively.

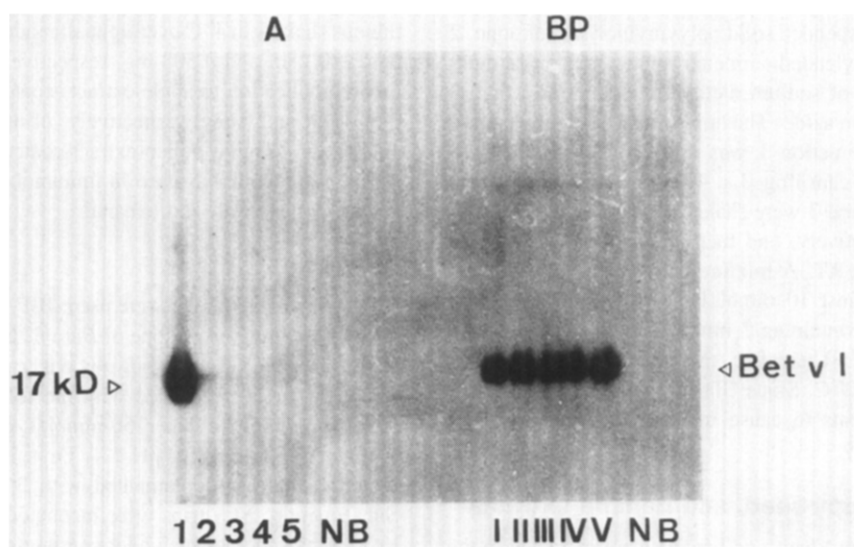


FIG. 3. Inhibition experiment. Sera from 32 patients allergic to birch pollen were pooled. Inhibition was performed by preincubation of this serum pool with native apple extract and birch-pollen extract, respectively, before immunoblotting procedure; *lanes 1 to 5*, sera, preincubated with 0, 0.0625, 0.125, 0.25, and 0.5 mg/ml of birch-pollen extract, reacting with apple extract-coated NC strips; *lanes I to V*: sera, preincubated with 0, 0.5, 1, 2, and 5 mg/ml of apple extract, reacting with birch-pollen extract-coated NC strips.

deionized formaldehyde.^{19, 20} Ten micrograms of birch-pollen RNA was used as a positive control. Thereafter, RNA was blotted to NC.²⁰

Preparation of the *Bet v I* coding cDNA

The complete *Bet v I* cDNA was ligated into plasmid SK⁺ Bluescript (Stratagene, La Jolla, Calif.; pBV1²¹). From

this construction, the complete *Bet v I* coding region was cut out with Eco R I and Hinf I; 100 ng of this fragment was labeled by random hexamer priming.²²

Prehybridization, hybridization, and washing

Prehybridization and hybridization were done, according to the method of Ausubel,²³ overnight at 37° C. Blots were

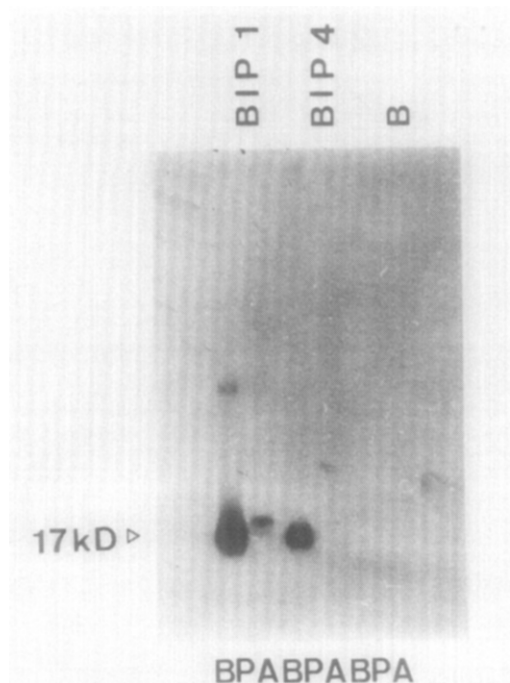


FIG. 4. Experiments with MAbs, raised against *Bet v I* (BIP 1 and BIP 4), reacting with birch pollen (BP) and apple (A) extract-coated NC strips.

washed twice in threefold concentrated SSC, the stock solution ($20 \times$ SSC) containing 3 mol/L of NaCl and 0.3 mol/L of sodium citrate, pH 7.0, and 0.1% of SDS. The washing procedure was performed for 10 minutes at RT, followed by two washings in $1.5 \times$ SSC, 0.1% SDS, and finally twice in $0.3 \times$ SSC, and 0.1% of SDS. During the last wash, temperature was increased to 50°C . Care was taken to avoid complete drying of the blots during the washing procedures. For exposure, Amersham Hyperfilm MP and Kodak intensifying screens were used.

RESULTS

IgE immunoblots

With birch-pollen extract, 81/83 patients (97.6%) with birch-pollen allergy demonstrated IgE binding to *Bet v I* (17 kD), and 73/83 patients (88%) reacted exclusively with this allergen (patients 1 to 5 and 8 and 9, Fig. 1.). In addition, 8/83 patients (9.6%) exhibited IgE binding to minor allergens (patients 6 and 7, Fig. 1). Two patients (2.4%) reacted exclusively with allergens in the range of 13 kD (patients 10 and 11, Fig. 1). The control group demonstrated no reactivity. All patients reacting with *Bet v I* demonstrated IgE binding to proteins of apple extract in the MW region of 17 and 18 kD (patients 1 to 9, Fig. 2); 7/83 (8.4%) revealed IgE binding to minor allergens of apple. Most patients reacting with an allergen in the region of 13 kD in the birch-pollen extract demonstrated a positive signal in this region with the apple

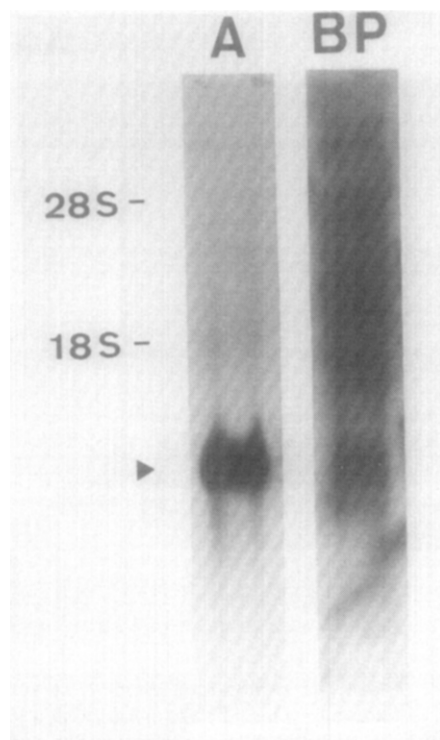


FIG. 5. Northern blot. lane A: 30 μg total apple RNA; lane BP: 10 μg of total birch-pollen RNA; the position of ribosomal bands is marked. An arrow indicates hybridization of the *Bet v I* cDNA with apple and birch-pollen mRNA at approximately 800 bases.

extract, also (5/7 patients, e.g., patients 7, 10, and 11, Figs. 1 and 2). By Western blot analyses, IgE binding was additionally found to other BPRF extracts (apple and hazelnut) in the region 17 kD but not to members of non-BPRF (poppy seed and banana) with a serum pool specific for *Bet v I* (see Fig. 6).

Inhibition experiments

A concentration of 0.0625 mg/ml of birch-pollen extract was sufficient to inhibit IgE binding to apple allergens (Fig. 3, A). In contrast, even 5 mg/ml of apple extract was not capable of weakening the IgE binding to *Bet v I* (Fig. 3, BP).

MAb blots

Two MAbs (BIP 1 and BIP 4) directed against distinct antigenic structures of *Bet v I*, 17 kD, were tested for reactivity with the apple extract. BIP 1 demonstrated binding to a 17 kD antigen in the apple extract, whereas with BIP 4, no binding to apple proteins could be observed (Fig. 4).

Northern blots

In Northern blot experiments, the whole *Bet v I* coding region (Eco RI/ Hinf I fragment) hybridized

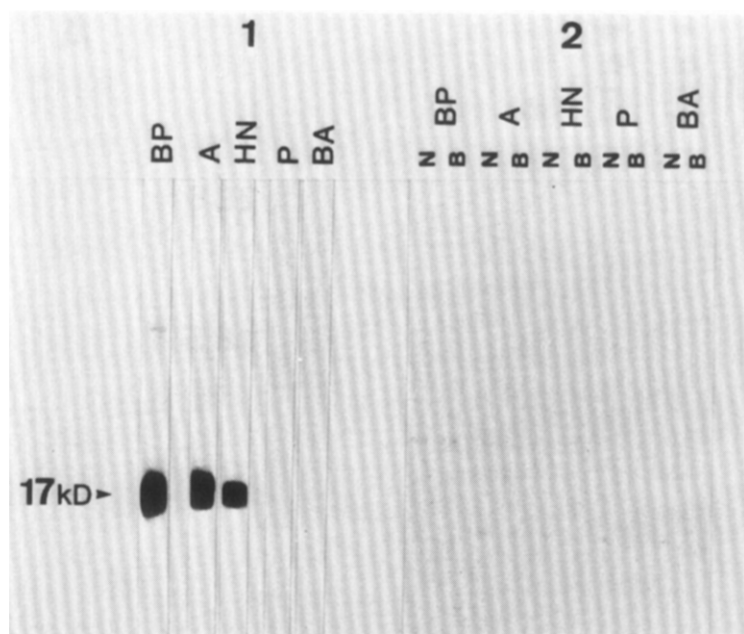


FIG. 6. IgE immunoblot. *Block 1:* sera from 32 patients allergic to birch pollen and reacting with the major allergen, *Bet v I* exclusively, were tested for reactivity with birch pollen (BP), apple (A), hazelnut (HN), poppy seed (P), and banana (BA) extracts, respectively; *block 2:* controls: a serum pool collected from nonatopic donors (N) and the buffer system (B) were tested for IgE reactivity with the above-mentioned protein sources.

with transcripts from apple under high stringency conditions at approximately 800 bases (Fig. 5).

DISCUSSION

Up to 70% of the patients suffering from birch-pollen allergy demonstrate an intolerance against a variety of raw fruits, vegetables, and nuts. Several publications dealt with this phenomenon,¹⁻¹¹ and many theories have been discussed in an attempt to explain these clinical observations. As yet, cross-reactivities of IgE Abs appear to be the most probable explanation for the observed clinical symptoms.

The aim of this study was to prove common allergenic structures in the birch pollen and apple proteins with Western and Northern blot techniques. It has been published in literature that many birch pollen-allergic patients demonstrate reactions after they have ingested apple.¹⁻¹⁰ The symptoms have mostly local character, but in several cases, systemic symptoms (like urticaria, angioedema, or nausea) were reported.

Our Western blot experiments revealed corresponding IgE binding in birch and apple extracts in the region of 17 kd; with the apple, in the form of a double band (17 and 18 kd) in 97% (81/83) of the sera tested. An individual threshold appears to exist for antibirch-pollen IgE titers to cause apple intolerance, since not all birch pollen-allergic patients suffer from apple

intolerance. (In our patients, collectively, 63/83 [75.9%] reported to avoid apples because of symptoms after ingestion.) Indications for this theory may be that in situations, when antibirch-pollen IgE Ab titers rise (e.g., at the beginning of immunotherapy or during the pollen season²⁴), a latent fruit intolerance frequently becomes manifest. However, we could identify a few patients with very high anti-*Bet v I* IgE titers not complaining of any symptoms after ingestion of apples (e.g., patient 8, Figs. 1 and 2). Two patients demonstrated no IgE binding to the 17 kd component, neither in the birch nor in the apple extract. In these cases, reactivities with birch pollen as well as apple extract occurred only at an MW range of about 13 kd (Figs. 1 and 2, patients 10 and 11).

These results strengthened our view that specific antibirch-pollen IgE Abs directed against the major allergen of birch pollen, *Bet v I*, or the minor allergen with MW 13 kd, cross-react with similar or even identical protein structures on the apple proteins.

Inhibition experiments demonstrated that sera preincubated with minimum amounts of birch-pollen extract lost their reactivity against apple allergens but that even high amounts of apple antigens were not able to inhibit the birch-pollen reaction significantly (Fig. 3). Similar observations were reported by other authors using RAST techniques.^{3, 6} The authors

claimed that all immunologic determinants in fruits and vegetables appear to be present also in birch pollen but not all birch-pollen determinants were present in fruits and vegetables.⁶ Our data support this theory. Furthermore, MAbs to *Bet v I* (BIP 1 and BIP 4¹⁶) were used to prove common antigenic structures (Fig. 4). One of the MAbs, BIP 1, demonstrated a distinctly positive reaction pattern with the apple extract in the same MW range as detected by human IgE, whereas a second MAb (BIP 4), obviously reacting with another epitope,¹⁷ was negative for apple proteins. These results again indicate (an) epitope(s) unique for *Bet v I* (BIP 4) and (an) additional epitope(s) common for birch pollen and apple (BIP 1).

In spite of the fact that birch and apple trees derive from unrelated plant species (birch, *Betulaceae*; apple, *Rosaceae*), the question rose whether the cross-reactivity refers to homologous proteins. Cloning and sequencing of a cDNA coding for the complete *Bet v I* molecule²¹ enabled us to apply DNA and RNA hybridization techniques. First, we used the Southern blot technique and found that the cDNA of the major allergen of birch pollen hybridized with restriction fragments of genomic DNA obtained from apple leaves (data not presented). Computer-aided sequence analyses on the DNA and protein level revealed significant identities/homologies of the *Bet v I* coding gene and *Bet v I* with "disease-resistance response genes" and "pathogenesis-related proteins," respectively, from *Pisum* (pea^{21,25}), *Petroselinum* (parsley²⁶), *Solanum* (potato²⁷), and *Collettrichum* (bean²⁸). These genes are constitutively transcribed and translated obviously in birch pollen only, but in other plant tissues, however, in response to stress conditions.²⁹ The sequence homologies on the protein level were all in the range of 70% (50% identity). Furthermore, these proteins were all of similar size (*Bet v I*, 159 amino acids versus 157, 154, 155, and 156 amino acids for pea, parsley, potato, and bean, respectively). From these data, it can be concluded that the *Bet v I* protein may fulfill a comparable function in birch trees. It is tempting to speculate that a *Bet v I* analogue in apples also represents a "disease-resistance response gene product," being expressed in fruits, a tissue with low metabolic activity but exposed to various stress conditions, in particular microbial attack. Northern blot experiments prove the transcription of a similar DNA sequence. The RNA of a questionable tissue (in this case, apple) is extracted and tested for hybridization with a known cDNA probe (*Bet v I*). Our Northern blot experiments demonstrated that the complete *Bet v I* cDNA hybridized under conditions of high stringency with mRNA from apple (Fig. 5) and therefore proved the existence of highly homologous tran-

scripts in birch pollen and apple. Furthermore, we could prove a *Bet v I* corresponding allergenic protein in other BPRFs (e.g., hazelnut) by means of IgE immunoblotting but not in some other plant tissues ("non-BPRF," poppy seed and banana; Fig. 6), suggesting that all nourishments belonging to this cluster of hypersensitivity contain proteins carrying similar epitopes accounting for crossreactions, which may cause clinical manifestations.

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