

Homology of the major birch-pollen allergen, *Bet v I*, with the major pollen allergens of alder, hazel, and hornbeam at the nucleic acid level as determined by cross-hybridization

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To investigate the relationship of the major allergens of birch (Bet v I), alder (Aln g I), hazel (Cor a I), and hornbeam (Car b I) at the nucleic acid level, a cDNA clone coding for the complete Bet v I protein was used for Northern and Southern blot experiments. RNAs were isolated from pollen of birch (Betula verrucosa), alder (Alnus glutinosa), hazel (Corylus avellana), and hornbeam (Carpinus betulus). Hybridization was performed at different stringencies. At high stringency, comparable binding of the complete Bet v I cDNA probe to pollen RNAs from birch, alder, and hazel could be observed, indicating high homology of the mRNAs coding for these allergens. With the 3' and 5' half fragments of the Bet v I cDNA, both probes bound to transcripts of all four tree pollens, but most strongly to birch RNA. In Southern blots, distinct binding patterns of genomic DNA digests of birch, alder, hazel, and hornbeam were observed. Most bands were observed with birch DNA digests and less with alder, whereas in genomic DNA digests of hornbeam and hazel, only one band was observed. The result of these cross-hybridization experiments indicate a high homology at the nucleic acid level of the four major allergens of trees belonging to the order Fagales. The sequence similarity presented here further corroborates earlier observations of immunologic cross-reactivity at the protein level. Therefore, in the case of the major allergens of the Betulaceae, an extract with only one major allergen, preferentially Bet v I, instead of all four major allergens, should be sufficient for diagnostic and therapeutic purposes. (J ALLERGY CLIN IMMUNOL 1991;87:677-82.)

Proteins isolated from aqueous extracts of pollen from birch, alder, hazel, and hornbeam were demonstrated to be responsible for most of the allergic symptoms (rhinitis, conjunctivitis, and pollen asthma) occurring in middle, eastern, and northern Europe, as well as in certain areas of North America during early and middle spring.¹

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Abbreviations used

cDNA: Complementary deoxyribonucleic acid

Bam H I, Eco R I: Restriction endonucleases

Eco R V, Hind III: Restriction endonucleases

Hinf I, Sac I: Restriction endonucleases

SSC: 3 mol/L of NaCl and 0.3 mol/L of sodium citrate, pH 7

SDS: Sodium dodecyl sulfate

PAGE: Polyacrylamide gel electrophoresis

kb: Kilobase

kbp: Kilobase pair

kd: Kilodalton

The molecular weight of the major allergens playing a predominant role in tree-pollen allergy, *Bet v I* (birch), *Aln g I* (alder), *Cor a I* (hazel), and *Car b I* (hornbeam), were determined to be approximately 16

to 20 kd, corresponding to approximately 160 to 180 amino acid residues.^{2,3} The isoelectric point of these allergens was in the range of 4 to 6. Characterization of these proteins by two-dimensional gel electrophoresis/immunoblotting demonstrated extensive cross-reactivity of patients' IgE.⁴ The monoclonal anti-*Bet v* I antibody, BIP 1, raised against *Bet v* I,⁵ strongly bound also to *Aln g* I, *Cor a* I, and *Car b* I.⁴

Partial N-terminal amino acid sequence analysis of *Bet v* I, *Aln g* I, *Cor a* I, and *Car b* I (40 to 45 amino acid residues) demonstrated high sequence identity-homology and putative common epitopes.^{3,6} However, nothing was known about the C-terminal parts of the allergens, which may be of immunologic relevance.

Recently, we isolated a complete cDNA coding for *Bet v* I from a lambda gt 11 expression library by screening with IgE from a selected patient.⁷ This cDNA clone enabled us to perform cross-hybridization experiments with pollen RNA and genomic DNA digests from alder, birch, hazel, and hornbeam.

The presence of a unique Bam H I restriction site in the *Bet v* I cDNA at base pair 399 (approximately in the middle of the exon(s) of the gene)⁷ facilitated the construction of two subclones in plasmid pUC 18, representing the respective 5' and 3' portion of the *Bet v* I cDNA. Thus, homologies in the C-terminal half of the protein could be investigated. Cross-hybridization of pollen RNA from alder, birch, hazel, and hornbeam was observed with the 5' and 3' probe, indicating sequence conservation over the complete allergenic molecules. Various restriction fragments of the respective genomic DNA digests hybridized with the *Bet v* I cDNA, corroborating the Northern blot experiments.

MATERIAL AND METHODS

Isolation and characterization of RNA

RNA was isolated from pollen purchased from Allergon AB, Engelholm, Sweden. The pollen was confirmed to be 95% pure by light and electron microscopy. One gram each of pollen of black alder (*Alnus glutinosa*), white birch (*Betula verrucosa*), hazel (*Corylus avellana*), and hornbeam (*Carpinus betulus*) was used for RNA extraction.

Each portion was homogenized separately with the addition of glass powder in a mortar under liquid nitrogen. Extraction was performed as previously described.⁸

Total RNA was characterized by 5% PAGE with a 16S and 23S molecular weight marker of defined concentration (2 µg/µl) (Boehringer Mannheim, Vienna, Austria).

Gels were stained with ethidium bromide to estimate the amount and quality of the RNA samples. Amounts of RNA were also estimated by spectrophotometry at a wavelength

of 260 nm. Ten micrograms of total RNA from alder, birch, hazel, and hornbeam were separated through a 1.1% denaturing agarose gel containing 6% deionized formaldehyde.^{9,10} One set of RNA and 10 µg marker were stained with ethidium bromide to ensure that similar amounts of intact RNA had been used.

Northern blotting

Identically prepared series of tree pollen RNAs were blotted onto nitrocellulose (Schleicher & Schuell BA 83, Kassel, Germany) according to previously published methods.¹¹

To achieve a constant ratio between amounts of ribosomal RNA and messenger RNA, total RNA was used for all Northern blots rather than poly A plus enriched RNA. Thus, information was obtained regarding the relative amount of specific mRNA in the preparations.

Isolation and purification of DNA

Young leaves from white birch, alder, hazel, and hornbeam were collected from trees in Maria Enzersdorf and Laxenburg, near Vienna. In case of alder, hazel, and hornbeam, the leaves were picked from one defined tree, whereas in case of birch, leaves were obtained from three defined trees. These leaves were immediately frozen in liquid nitrogen and stored at -70°C until use.

DNA extraction was performed essentially according to the method of Dellaporta et al.¹² DNA was then purified by CsCl gradient centrifugation according to the method of Maniatis.¹² Integrity and amount of DNA was estimated by agarose gel electrophoresis (0.8% agarose gel; ethidium bromide staining).

Restriction digest, agarose gel electrophoresis and Southern blotting of DNA

Ten micrograms of DNA from each alder, birch, hazel, and hornbeam were digested with 10 units of Eco R V and Hind III, respectively (Boehringer Mannheim), according to the manufacturer's advice.

Digested DNA was separated by agarose gel electrophoresis (0.8% agarose) and blotted to nitrocellulose BA 83 from Schleicher & Schuell.¹³

Construction of subclones containing the 5' and 3' half of the *Bet v* I cDNA

We subcloned the complete *Bet v* I cDNA, flanked by lambda gt 11 sequences into the Kpn I and Sac I site of plasmid Sk⁺ bluescript.⁷ From this construction, designated pBV 1, the complete *Bet v* I coding region was excised.

The presence of a unique Bam H I site at base pair 399 of the *Bet v* I cDNA allowed the construction of a subclone representing the 3' coding regions, named p*Bet v* I-Bam 3' and a second one representing the 5' half of the *Bet v* I cDNA, together with its lambda gt 11 flanking region. The second subclone was named p*Bet v* I-Bam 5'.¹⁴

All subcloning steps were done essentially according to

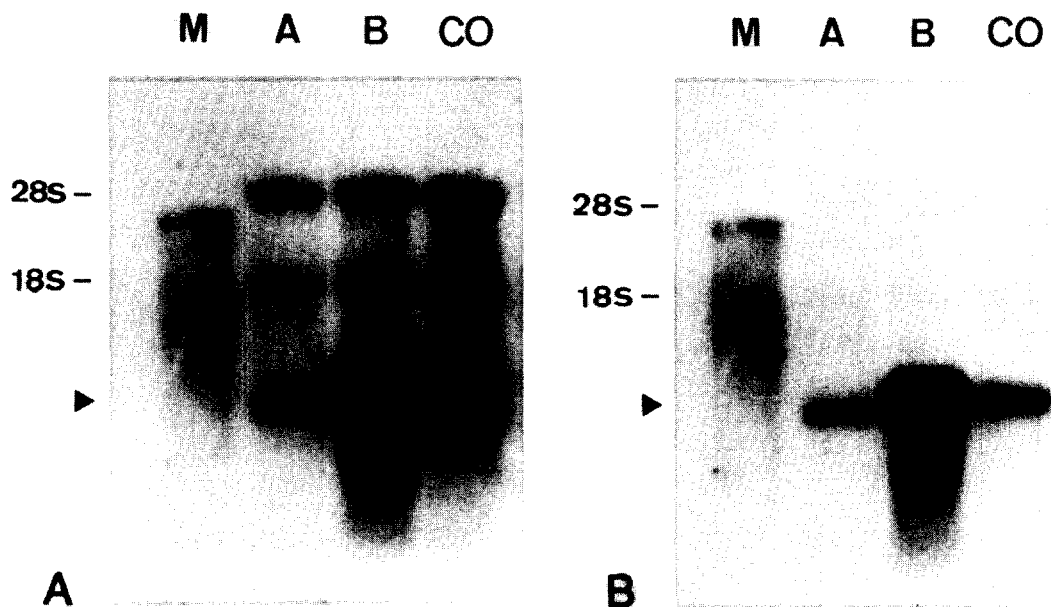


FIG. 1. A, Northern blot: 23S/16S ribosomal weight marker from *Escherichia coli* (lane M); total pollen RNA from black alder (lane A), white birch (lane B), and hazel (lane CO) was hybridized with the complete *Bet v I* cDNA (Hinf I/Eco R I fragment). Position of hybridizing transcripts is marked with an arrow (~0.8 kb). The 28S and 18S ribosomal bands indicate that comparable amounts of total RNA were blotted to nitrocellulose. Washing conditions: $1.5 \times$ SSC and 0.1% SDS at 35° C. B, The same Northern blot as in A was washed up to $0.3 \times$ SSC and 0.1% SDS at 50° C; 28S and 18S ribosomal bands disappear, whereas signals of specifically hybridizing transcripts remain equally intensive, as in A.

previously published methods.¹⁵ As a control, *pBET v I-Bam 3'* was digested with Eco R I/Bam H I and *pBet v I-Bam 5'* with Sac I/Bam H I. The size of these fragments was analyzed by agarose gel electrophoresis.

Preparation of fragments for random-primed DNA labeling

The complete *Bet v I* cDNA was excised from *pBV 1* with Hinf I and Eco R I. In parallel, the 5' region (Hinf I/Bam H I) and 3' region (Bam H I/Eco R I) were isolated from the above described subclones *pBet v I-Bam 5'* and *pBet v I-Bam 3'*. All fragments were eluted from 1% agarose gels.¹⁵ One hundred nanograms of each fragment was labeled with alpha ³²P-dCTP by randomly primed DNA labeling.¹⁶ Nonincorporated nucleotides were separated from labeled DNA with nick columns (Pharmacia, Uppsala, Sweden).

Prehybridization, hybridization, and washing of Northern and Southern blots

Prehybridization and hybridization solutions were prepared according to the method of Ausubel.¹⁵ The hybridization mixture contained 10% (wt/vol) dextran sulfate 5000 (Sigma Chemical Co., St. Louis, Mo.) to enhance RNA/DNA and DNA/DNA interaction. Prehybridization and hybridization was performed overnight at 37° C. Ra-

diolabeled probe (10⁷ cpm, Cerenkow radiation) was added. Blots were washed twice in $3 \times$ SSC and 0.1% SDS at room temperature for 10 minutes, followed by washing twice in $1.5 \times$ SSC and 0.1% SDS, and finally twice in $0.3 \times$ SSC and 0.1% SDS. During the last wash, temperature was increased to 50° C.

Care was taken to avoid complete drying of the blots to make further washing possible. For exposure, Hyperfilm MP (Amersham International, Buckinghamshire, England) and intensifying screens (Kodak, Heidelberg, Germany) were used.

RESULTS

Northern blots

RNA from alder, birch, and hazel was hybridized with the complete *Bet v I* cDNA (Hinf I/Eco R I fragment of *pBV 1*) applying two different stringency conditions ($1.5 \times$ SSC and 0.1% SDS at 35° C, Fig. 1, A; $0.3 \times$ SSC and 0.1% SDS at 50° C, Fig. 1, B). At low stringency, hybridization to a band of approximately 0.8 kb was observed with pollen RNA from all trees, most notably with birch (Fig. 1, A). At high stringency, the same binding of the *Bet v I* cDNA probe to the RNAs could be observed (Fig. 1, B), indicating high homology of the major

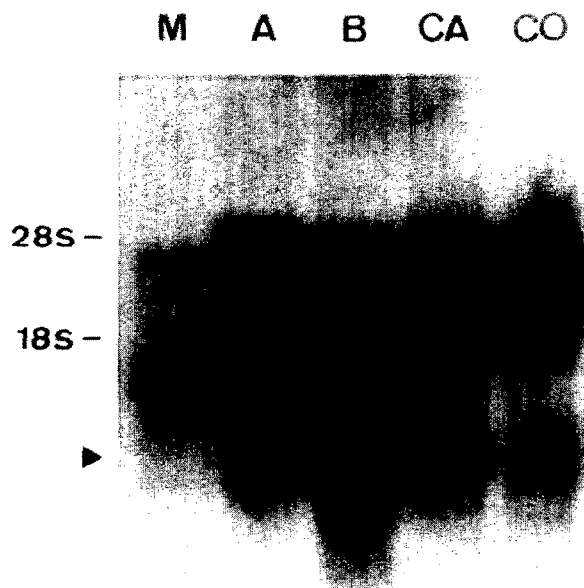


FIG. 2. Northern blot: 23S/16S ribosomal weight marker from *Escherichia coli* (lane M); total pollen RNA from black alder (lane A), white birch (lane B), hornbeam (lane CA), and hazel (lane CO) was hybridized with the 5' portion of the *Bet v I* cDNA (Bam H I/Hinf I fragment); 28S and 18S ribosomal bands indicate that comparable amounts of RNA were blotted to nitrocellulose. All tree-pollen mRNAs hybridized with the probe at approximately 0.8 kb, as indicated by an arrow.

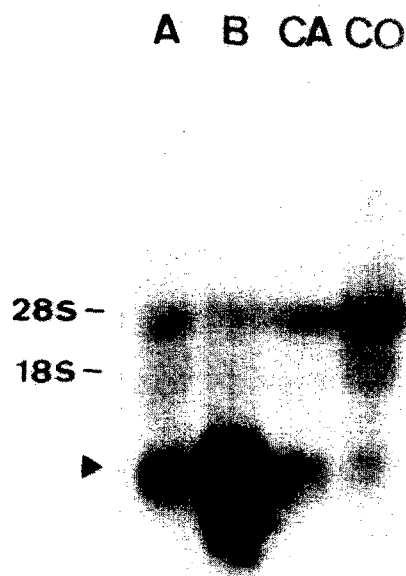


FIG. 3. Northern blot: total pollen RNA from black alder (lane A), white birch (lane B), hornbeam (lane CA), and hazel (lane CO) was hybridized with the 3' portion of the *Bet v I* cDNA (Bam H I/Eco R I fragment). The position of the 28S and 18S ribosomal bands is marked. The arrow points to the hybridizing transcripts from all trees (~0.8 kb).

tree-pollen allergens at the nucleic acid level. In addition, at low stringency washing conditions, non-specific hybridization with the 28S and 18S ribosomal RNAs was observed (Fig. 1, A). Nonspecific hybridization of the pollen with ribosomal RNAs indicated that similar amounts of RNA had been loaded onto the gel (Fig. 1, A).

RNA from alder, birch, hazel, and hornbeam was hybridized with the 5' and 3' portion of the *Bet v I* cDNA (Bam H I/Hinf I fragment of *pBet v I*-Bam 5' and Bam H I/Eco R I fragment of *pBet v I*-Bam 3'). As illustrated in Figs. 2 and 3, both DNA probes bound to mRNA from all tree pollens, but most strongly to birch mRNA (washing condition: $1 \times$ SSC and 0.1% SDS at 45°C). Again, hybridization of the ribosomal bands indicates that comparable amounts of RNA had been blotted to nitrocellulose.

Southern blots

Genomic DNA from alder, birch, hazel, and hornbeam was digested with Eco R V and Hind III. DNA digests were hybridized with the complete *Bet v I* cDNA (Eco R I/Hinf I fragment of *pBV I*).

The probe bound to at least four bands (Eco R V) and nine bands (Hind III), respectively, in birch DNA digests (Fig. 4, A). In the case of hazel and hornbeam, genomic DNA digests (Eco R V and Hind III), only one band was observed, whereas at least three bands (Eco R V) and five bands (Hind III) could be observed in genomic blots of alder (Fig. 4, B).

DISCUSSION

Computer-aided sequence analyses of the deduced amino acid sequence of *Bet v I* (159 amino acids) revealed putative antibody binding epitopes on the N- and well as the C-terminus of the molecule.⁷ Since sequence homology of *Bet v I* to a protein involved in disease resistance of parsley and pea exists,^{17, 18} it would be expected to have high homology at the level of RNA and DNA sequence to the closely related trees of the order Fagales, alder, hazel, and hornbeam.

In Northern blots, all probes reacted most strongly with pollen RNA isolated from birch. This finding appears not to be due to different amounts of total RNA applied to the gel. Conversely, the relative amount of pollen mRNA coding for *Aln g I*, *Car b I*.

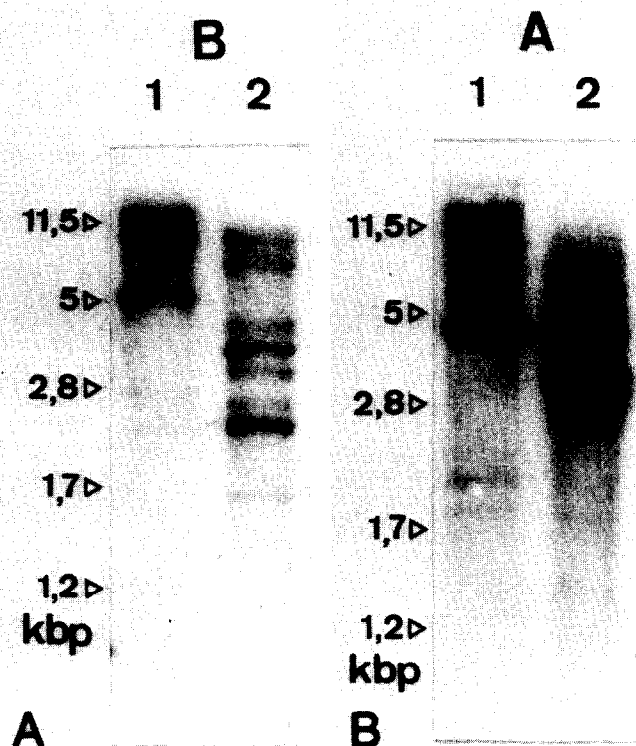


FIG. 4. A. Southern blot: four bands in Eco R V digests (1) and nine bands in Hind III digests (2) of genomic DNA from birch, (B), hybridized with the complete *Bet v I* cDNA. **B.** Southern blot: three bands in Eco R V digests (1) and four bands in Hind III digests (2) of genomic DNA from alder (A), bound to the *Bet v I* cDNA.

and *Cor a I* appears to be lower than the amount of the *Bet v I* coding mRNA. Thus, our data demonstrate sequence conservation of the mRNAs coding for these major tree-pollen allergens.

Markedly distinct patterns were observed in Southern blots with Eco R V and Hind III digests of birch, alder, hazel, and hornbeam genomic DNA and the complete *Bet v I* cDNA as probe. Most bands were observed with DNA from birch and less with alder DNA, whereas with DNA from hazel and hornbeam only, one band could be observed. All these Southern blot data are in best accordance with the Northern blot experiments. The presence of restriction fragments in digests of genomic DNA from alder, hazel, and hornbeam, which cross-hybridize with the *Bet v I* cDNA, documents the homology of the respective major tree-pollen allergens also at the DNA level.

It is well-known that the three-dimensional structure of proteins is more conserved than the amino acid sequence and the nucleotide sequence. Therefore, the fact that there is high homology within the major allergens of trees belonging to the order Fagales, demonstrated by cross-hybridization experiments at

the nucleic acid level comprising 5' and 3' regions of the exons of the genes, indicates highly conserved protein structure spanning the complete allergen molecules.

For this reason, our data support the idea that, in case of the major allergens of birch, alder, hazel, and hornbeam, cross-sensitization, elicited probably in most cases by birch pollen, is to be expected. This concept is supported by clinical studies demonstrating that immunotherapy with birch-pollen extract alone is effective in reducing IgE responses (RAST measurements) as well as skin and nasal sensitivity in patients allergic to pollen of alder, birch, hazel, and hornbeam.¹⁹⁻²¹ Therefore, it would be expected that for diagnostic and therapeutic measures, at least in the case of the major allergens, a preparation containing only the extract from one plant, preferentially birch, should be sufficient.

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REFERENCES

1. Yman L. Botanical relations and immunological cross-reactions in pollen allergy. 2nd ed. Uppsala, Sweden: Pharmacia AB, 1982.
2. Ipsen H, Bowadt H, Janniche H, et al. Immunochemical characterization of reference alder (*Alnus glutinosa*) and hazel (*Corylus avellana*) pollen extracts and the partial immunochemical identity between the major allergens of alder, birch, and hazel pollen. *Allergy* 1985;40:510-8.
3. Ipsen H, Hanssen O. Structural similarities between amentiferous tree pollen major allergens [Abstract]. *Allergy* 1988; 43(suppl 7):B-11.
4. Rumpold H, Rohac M, Bohle B, Breitenbach M, Scheiner O, Kraft D. The relationship of *Bet v* I epitopes recognized by patients' IgE and monoclonal anti-*Bet v* I antibodies. In: Sehon A, Kraft D, Kunkel G, eds. Epitopes of atopic allergens. Brussels, Belgium: UCB Institute of Allergy, 1990:26-8.
5. Jarolim E, Tejkl M, Rohac M, et al. Monoclonal antibodies against birch pollen allergens: characterization by immunoblotting and use for single-step affinity purification of the major allergen *Bet v* I. *Int Arch Allergy Appl Immunol* 1989;90:54-60.
6. Vik H, Florvaag E, Dybendal T, Omnaas E, Holen E, Elsayed S. The biological activities of solid phase synthetic allergenic epitopes from major allergens of tree pollen [Abstract]. *Allergologie* 1989;B 1366 E:WSO2.13.
7. Breiteneder H, Pettenburger K, Bito A, et al. The gene coding for the major birch pollen allergen, *Bet v* I, is highly homologous to a pea disease resistance response gene. *EMBO J* 1989;8:1935-8.
8. Breiteneder H, Hassfeld W, Pettenburger K, et al. Isolation and characterization of messenger RNA from male inflorescences and pollen of the white birch (*Betula verrucosa*). *Int Arch Allergy Appl Immunol* 1988;87:19-24.
9. Thomas PS. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc Natl Acad Sci USA* 1980;77:5201-5.
10. Lehrach H. RNA molecular weight determinations by gel-electrophoresis under denaturing conditions: a critical reexamination. *Biochemistry* 1977;16:4743-51.
11. Dellaporta SL, Wood J, Hicks JB. A plant DNA miniprep: version II. *Plant Molec Biol Reporter* 1983;1:19-21.
12. Maniatis T. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory, 1982.
13. Southern EM. Detection of specific sequences among DNA fragments separated by gelelectrophoresis. *J Mol Biol* 1975; 98:503-17.
14. Valenta R, Breiteneder H, Pettenburger K, et al. RNA and DNA sequence similarities of the major allergens of birch, alder, hazel, and hornbeam [Abstract]. *Allergologie* 1989;B 1366 E:WSO2.20.
15. Ausubel FM. Current protocols in molecular biology. New York: Green Publishing Associates and Wiley-Interscience, 1987.
16. Feinberg AP, Vogelstein B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 1983;132:6-13.
17. Fristensky B, Horowitz D, Hadwiger LA. cDNA sequences for pea disease resistance response genes. *Plant Mol Biol* 1988;11:713-5.
18. Somssich IE, Schmelzer E, Kawalleck E. Gene structure and in situ transcript localization of pathogenesis related protein 1 in parsley. *Mol Gen Genet* 1988;213:93-8.
19. Nüchel Petersen B, Janniche H, Munch EP, et al. Immunotherapy with partially purified and standardized tree pollen extracts. *Allergy* 1988;43:353-62.
20. Wihl JA, Ipsen H, Nüchel Petersen B, Munch EP, Janniche H, Loewenstein H. Immunotherapy with partially purified and standardized tree pollen extracts. *Allergy* 1988;43:363-9.
21. Ipsen H, Schwartz B, Wihl JA, et al. Immunotherapy with partially purified and standardized tree pollen extracts. *Allergy* 1988;43:370-7.