

DISTRIBUTION OF ALLERGENS AND ALLERGEN-CODING mRNAs IN VARIOUS TISSUES OF WHITE BIRCH

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(First received 18 December 1990; accepted in revised form 13 March 1992)

Abstract—The distribution of allergenic proteins was investigated in various tissues of white birch, *Betula verrucosa* (pollen, leaves and male inflorescences containing immature pollen). In addition, callus and suspension culture cells were investigated for expression of IgE-binding proteins. Furthermore, RNA was extracted from all these tissues and subjected to *in vitro* translation in a cell-free wheat germ system. *Bet v* I, the major birch pollen allergen, could be extracted easily from pollen, and in low amounts from callus and leaves. No *Bet v* I could be extracted from immature male inflorescences. Minor allergens were expressed in high concentrations in pollen and in low concentrations in immature male inflorescences. No minor allergens could be detected in callus and leaves. In contrast to these observations, RNA from all the tissues as well as from callus could be translated *in vitro* into *Bet v* I as well as into minor allergens, in particular birch profilin (*Bet v* II), an important minor allergen. These data suggest that IgE-binding proteins of *B. verrucosa*, especially *Bet v* I, under certain circumstances can readily be synthesized in tissues other than pollen. This concept is corroborated by the recent observation that *Bet v* I reveals high homology with disease resistance response gene products from other plants, suggesting a similar function of *Bet v* I for the birch.

INTRODUCTION

Tree pollen allergens range third in the list of inhalant allergens (after mites and grass pollens), being responsible for immediate type hypersensitivity reactions in large parts of the Northern hemisphere and, recently, also in Australia (Hemmens *et al.*, 1988; Jarolim *et al.*, 1989a).

Most of the airborne allergens (pollens, mite faeces, animal dander, mould spores) are proteins or glycoproteins of molecular weights in the range of 13–50 kD. Their ready solubility in aqueous systems, or even protease activities (Stewart *et al.*, 1990), allows them to readily intrude through the mucous barriers of the respiratory pathways, evoking the IgE-mediated release of inflammatory mediators (histamine, arachidonic acid metabolites and others), thus causing the well-known symptoms of allergic rhinitis, rhinoconjunctivitis and *Asthma bronchiale*. Furthermore, many patients suffering from birch pollinosis show hypersensitivity to certain fruits like apples and nuts (Eriksson *et al.*, 1982; Halmeperu and Loewenstein, 1985; Calkhoven *et al.*, 1987). Recently, we could find a molecular basis for these clinical observations: a cDNA coding for *Bet v* I hybridized in Northern blot experiments with RNA extracted from apple fruits. A monoclonal antibody (BIP 1; Jarolim *et al.*, 1989b) raised against *Bet v* I reacted with a 17 kD protein from apples, suggesting that *Bet v* I and the apple allergen possess common IgE-binding structures (Ebner *et al.*, 1991). These observations indicate that *Bet v* I-homologues

may be found apart from pollen in non-germinative tissues.

More than 50 allergens have been purified and characterized so far by either conventional biochemical methods (Mole *et al.*, 1975; Marsh *et al.*, 1987; Chapman *et al.*, 1987; Chapman *et al.*, 1988; Klapper *et al.*, 1988) or, more recently, by molecular cloning techniques (Scheiner *et al.*, 1992). Nevertheless, little is known presently about biochemical functions of these allergens except for *Der p* I and *Der f* I: cloning and sequencing (Chua *et al.*, 1988) revealed significant homologies with thiol proteases (papain, cathepsin), which could be confirmed using biochemical methods (Stewart *et al.*, 1990).

In a recent paper, we published the characterization of a cDNA coding for the complete major allergen of white birch, *Bet v* I (Breiteneder *et al.*, 1989). A 55% sequence identity and a 70% homology with a disease resistance response protein from *Pisum sativum* was found (Breiteneder *et al.*, 1989). Other striking identities and homologies with pathogenesis-related (PR) proteins from parsley (Somssich *et al.*, 1988; 42% identity), from potato (Matton and Brisson, 1989; 46% identity) and from bean (Walter *et al.*, 1990; 44.8% identity) were obtained by computer-aided sequence comparisons. PR proteins are known to be synthesized in response to microbial pathogens (e.g. *Pseudomonas* sp., *Fusarium* sp.; Daniels *et al.*, 1987), heavy metal ions and other environmental stress as described in detail by Lamb *et al.* (1989), Taylor *et al.* (1990) and van de Loeht *et al.* (1990).

So far, nothing is known about the biological functions of *Bet v* I in the birch plant. Singular observations of plant allergens found in stems and leaves have been made before (Shafiee *et al.*, 1973; Lahti and Hannuksela, 1980). Recently, a further birch pollen allergen was cloned and sequenced and characterized as the actin binding protein, profilin, in our laboratory (Valenta *et al.*, 1991a). This 14 kD protein was designated *Bet v* II (Valenta *et al.*, 1991a; GenBank: accession number M65179) an ubiquitous component of the eucaryotic cytoskeleton. Although only 9% of birch pollen-allergic patients react with *Bet v* II, profilins from other pollens seem to be important allergens (Valenta *et al.*, 1991b; Valenta *et al.*, 1992). Taking these data into account, it was of interest to study protein expression of *Bet v* I and *Bet v* II (birch profilin) not only in pollen but also in various other tissues of white birch.

Proteins were extracted from pollen, male inflorescences, leaves and undifferentiated tissue culture cells (callus). These materials were investigated for tissue-specific expression of these allergens by means of immunoblotting, using sera from selected patients with distinct IgE-binding patterns to birch pollen allergens (Breiteneder *et al.*, 1988; Jarolim *et al.*, 1989a). In addition, RNA extracted from the various tissues was tested for transcripts coding for *Bet v* I and minor allergens by *in vitro* translation in a cell-free wheat germ system. The translated proteins were separated by SDS-PAGE and characterized by immunoblotting, using the above mentioned selected patients' sera.

MATERIALS AND METHODS

Biological material

Immature male inflorescences from *Betula verrucosa* (BV) from two selected trees (Maria Enzersdorf, near Vienna) were collected in December and January. The specimens were immediately frozen in liquid nitrogen and were maintained at -70°C until use. Pollen of BV was purchased from Allergon AB (Engelholm, Sweden). Young leaves from BV were collected from two selected trees in Maria Enzersdorf (see above) and from two selected trees on a traffic island in downtown Vienna. Solid callus tissue was obtained from seedlings according to basic plant-tissue culture methods (Huang and Murashige, 1977; Tremblay *et al.*, 1985). The seedlings were derived from the above mentioned trees (Maria Enzersdorf).

Sera

Sera from 11 patients allergic to birch pollen proteins were selected according to case history, skin prick tests and RAST. None of these patients had received immunotherapy. One group of patients exhibiting IgE-binding exclusively to *Bet v* I as shown in immunoblotting experiments were pooled (pool C). Furthermore, two selected sera, one from a patient revealing IgE-binding to pollen proteins with mol. wts ranging from 13–68 kD (serum D), the other one reacting exclusively with *Bet v* II (serum B)—as shown in immunoblotting

experiments—were also used for the experiments described. Sera from eight healthy laboratory staff members without any apparent type I allergy (skin prick tests, RAST, case histories) were pooled into a NHS pool (pool A).

Extraction of plant tissue proteins

Protein extraction of pollen and callus tissue was done according to Breiteneder *et al.* (1988). Leaves and male inflorescences were homogenized in liquid nitrogen and protein extraction was performed as described (Björkstén *et al.*, 1980) with some modifications.

Preparation of RNA

RNA from male inflorescences and pollen was extracted as previously described (Breiteneder *et al.*, 1988). RNA from leaves and callus was extracted using a buffer containing 2% (w/v) cetyltrimethylammoniumbromide, 100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, pH 7.8. Messenger RNA (mRNA) was enriched by passing the RNA over oligo-dT-cellulose (Sigma, St Louis, U.S.A.).

In vitro translation

RNA from pollen, leaves, male inflorescences, and callus was translated in a cell-free wheat germ system (RPN.1, Amersham International, England) according to instructions provided by the manufacturer. The translated proteins were separated by SDS-PAGE and subjected to immunoblotting as described (Breiteneder *et al.*, 1988).

RESULTS

Single sera/serum pools were tested for their IgE-binding patterns by immunoblotting of aqueous birch pollen extracts, as shown in Fig. 1.

IgE-binding of aqueous extracts from birch-pollen, male inflorescences, callus and from leaves of non-stressed plants (Maria Enzersdorf), respectively, are shown in Fig. 2, using the selected sera and serum-pools A, B, C and D as described in Materials and Methods: strong IgE-binding to a 17 kD protein (*Bet v* I) can be observed with pollen, weaker binding with callus tissue and with leave extracts, when probed with the serum pool recognizing primarily *Bet v* I (pool C) and the single serum (D), respectively. IgE-binding to *Bet v* I could not be detected with male inflorescences containing immature pollen. IgE-detection of *Bet v* II (birch profilin); was seen only with pollen and callus tissue, either as a single band (B) or together with other allergens (D). *Bet v* II could not be detected in immature male inflorescences (lanes 2, 3). Minor allergens in the upper mol. wt range can be found in pollen (from 30 to 68 kD), in immature male inflorescences (from 44 to 85 kD), and one single allergen of approximately 75 kD in callus tissue. The NHS-serum pool revealed no reactivity to any tissue extract (panel A).

IgE-binding proteins which were translated *in vitro* from mRNA-preparations extracted from immature male inflorescences, pollen, callus, and leaves of non-

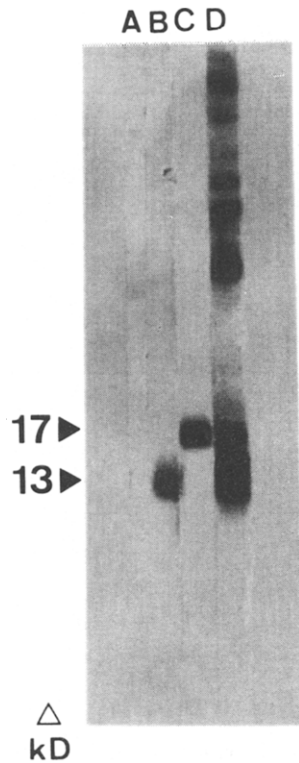


Fig. 1. An aqueous extract of pollen proteins was separated on an SDS-PAGE and immunoblotted. Single sera/serum pools were tested for their IgE-binding patterns to birch pollen proteins with ^{125} I-labelled anti-human IgE. Lane A: NHS-serum pool; lane B: single serum showing IgE-binding selectively to *Bet v* II; lane C: serum pool of 11 patients allergic to birch pollen, showing IgE-binding exclusively to *Bet v* I; lane D: single serum, showing IgE-binding to birch pollen proteins in a range of 13–68 kD.

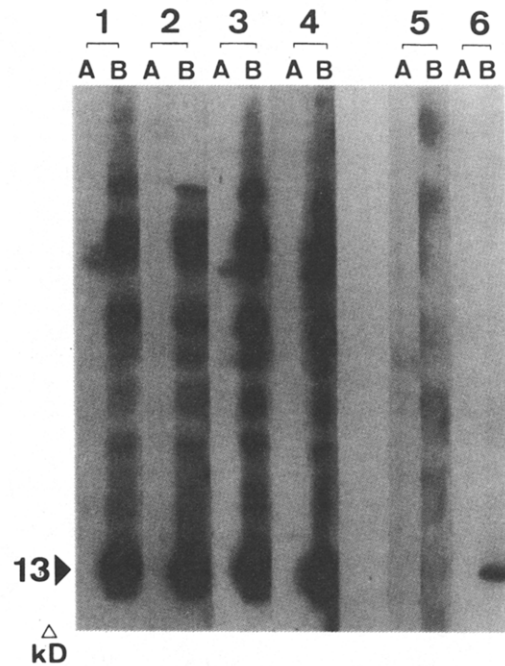


Fig. 3. *In vitro*-translation of mRNA-preparations from pollen (panel 1), leaves (panel 2), callus (panel 3), male inflorescences (panel 4), wheat germ system without addition of RNA (negative control, panel 5). Block 6: aqueous pollen extract (positive control). Synthesized proteins were separated by SDS-PAGE and immunoblotted with the following sera: lanes A: NHS pool, lanes B: single serum binding selectively to *Bet v* II in aqueous pollen extracts. IgE-binding was detected with ^{125} I-labelled anti-human IgE.

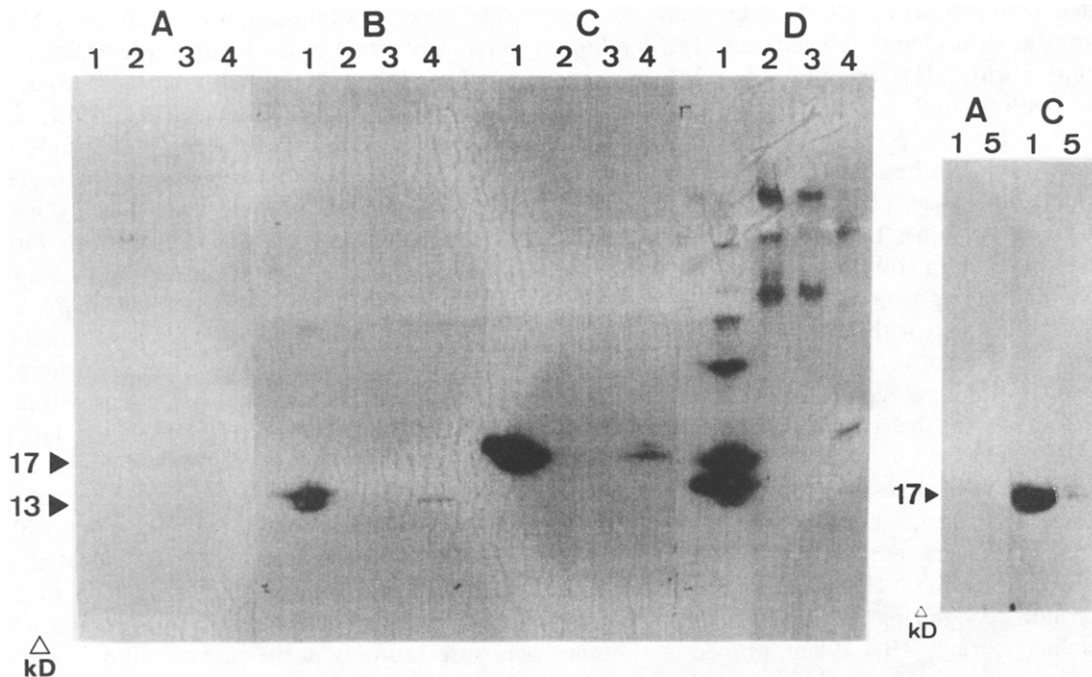


Fig. 2. Aqueous extracts of various tissues of *BV* were subjected to SDS-PAGE and immunoblotting. Lanes 1: pollen; lanes 2 and 3: male inflorescences; lanes 4: callus; lanes 5: leaves. Panels A: NHS-serum pool; panel B: single serum, showing IgE-binding to *Bet v* II in aqueous pollen extracts; panels C: serum pool showing IgE-binding exclusively to *Bet v* I; panel D: single serum showing IgE-binding to birch pollen proteins in a range from 13–68 kD. IgE-binding was detected with ^{125} I-labelled anti-human IgE.

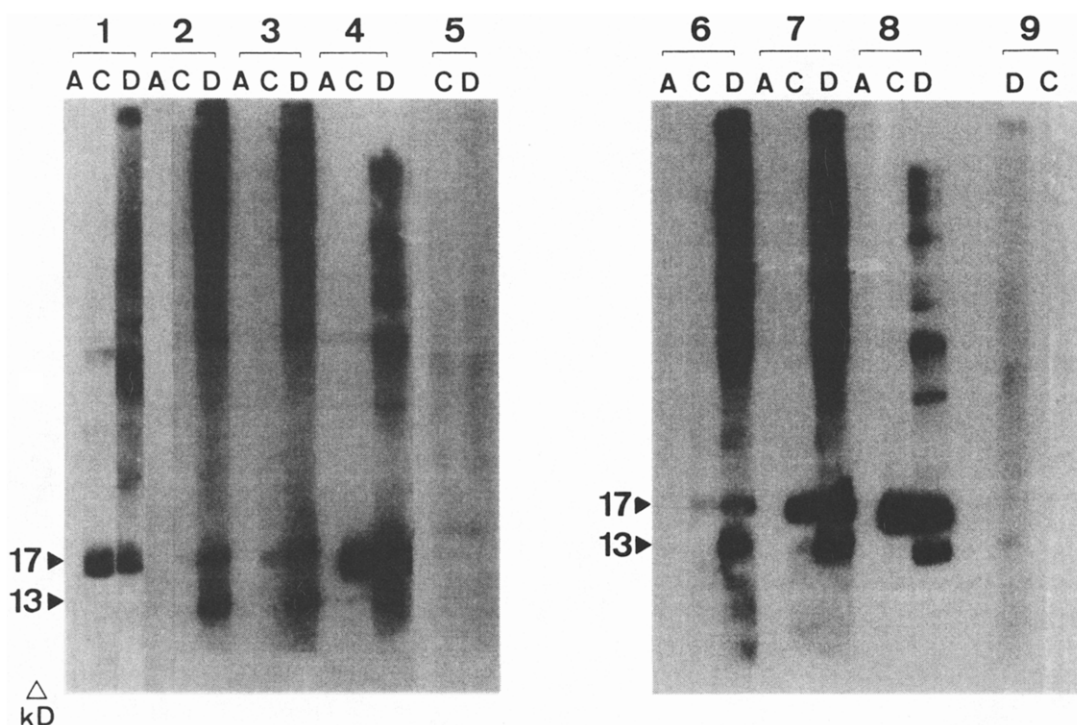


Fig. 4. *In vitro*-translation of mRNAs from male inflorescences, (panel 1), pollen (panel 2, 3), callus (panel 6) and leaves (panel 7). Panels 4 and 8: aqueous pollen extract (positive control), panels 5 and 9: wheat germ system without addition of RNA (negative control). Synthesized proteins were separated by SDS-PAGE and immunoblotted with the following sera: lanes A: NHS pool; lanes C: serum pool binding to *Bet v I* in aqueous pollen extracts; lanes D: single serum binding to proteins in a range from 13–68 kD in aqueous pollen extracts. IgE-binding was detected with ^{125}I -labelled anti-human IgE.

stressed plants are shown in Fig. 4. The synthesized proteins were separated by SDS-PAGE and immunoblotted with the sera A, C and D. None of the *in vitro*-translated proteins showed any IgE-binding when incubated with NHS-serum pool (A). IgE-binding to proteins with a mol. wt of 17 kD was observed when probed with serum pool C. After incubation with serum D, *in vitro*-synthesized proteins gained from immature male inflorescences showed no IgE-binding in the 13 kD range. All other tissues revealed strong IgE-binding at 13 kD and, in addition, IgE-binding at 17 kD and at mol. wts ranging from 30 to 68 kD. An aqueous pollen extract incubated with sera A, C and D respectively, served as a positive control (blocks 4 and 8). Virtually no IgE-binding could be detected without addition of RNA to the *in vitro* translation assays (blocks 5 and 9).

In Fig. 3, *in vitro*-synthesized proteins gained from RNA extracts of pollen, leaves, callus and immature male inflorescences were subjected to SDS-PAGE and immunoblotted using the NHS-serum pool (A) and the selected serum, recognizing exclusively *Bet v II* in aqueous pollen extracts (B). When probed with this serum, translated proteins from all tissues showed strong IgE-binding at 13 kD with additional strong IgE-binding in the upper mol. wt-ranges. A control without addition of RNA showed no IgE-binding after incubation with the NHS-serum pool (A) and only weak bands at 30–90 kD, when probed with serum B.

DISCUSSION

The presence of the major allergen, *Bet v I*, and minor allergens of white birch in aqueous extracts from pollen investigated by CRIE and immunoblotting has been described in detail (Apold *et al.*, 1981; Ipsen and Loewenstein, 1983; Tejkl *et al.*, 1988; Jarolim *et al.*, 1989a). These results were confirmed by corresponding experiments on the nucleic acid level by means of *in vitro*-translation of polyA⁺ RNA from birch pollen (Breiteneder *et al.*, 1988). Considering that a previously cloned and sequenced cDNA coding for *Bet v I* showed high homology to plant pathogenesis-related (PR) proteins (Breiteneder *et al.*, 1989), the distribution and the function of *Bet v I* and minor allergens within the birch plant seemed to be of interest. So far, the data presented in this paper point at the following facts: aqueous extracts of birch pollen contain IgE-binding proteins in the mol. wt range from approximately 10 to 70 kD (Figs 1 and 2). As for the other tissues investigated, IgE-binding to *Bet v I* could in fact be shown in callus and non-stressed leaf tissue. Weak IgE-binding to *Bet v II* was observed only in the case of callus tissue but not in other plant tissues. Sera exhibited IgE-binding to immature male inflorescences only at mol. wts ranging from 44–90 kD, but neither to *Bet v I* nor *Bet v II* (Fig. 2). This leads to the conclusion that, by protein extraction, we do find *Bet v I* and minor allergens, in particular *Bet v II*, in birch tissues other than pollen, yet to a much

lesser extent compared to the amount of these allergens in pollen. In addition, there appears to exist a particular tissue-specific expression: *Bet v I* and *Bet v II* were not expressed in immature male inflorescences containing pollen at the tetrad and microspore stage. This suggests that allergens are expressed only at a late stage of pollen development. Moreover, the callus tissue was shown to serve as a suitable *in vitro*-system for studies of the expression of proteins and thus of possible links between PR-proteins and allergens of birch. In contrast to these protein extraction data, *in vitro*-translation of mRNA extracted from the same materials yielded clearly detectable amounts of IgE-binding proteins (Figs 3 and 4). The presence of mRNA coding for the IgE-binding proteins, in particular *Bet v I*, in all the tissues may suggest control of *Bet v I*-expression, and possibly of minor allergens, on the translational or even post-translational level. It may well be that *Bet v I* is a member of a gene family (Rohac *et al.*, 1991; Valenta *et al.*, 1991c) that includes PR proteins that have been shown to be highly homologous to *Bet v I*: 70% homology with a pea disease resistance protein (Fristensky *et al.*, 1988; Breiteneder *et al.*, 1989); 60% homology with a PR-proteins of parsley, potato and bean, respectively (Somssich *et al.*, 1988; Matton and Brisson, 1989; Walter *et al.*, 1990).

This homology leads to the question of whether there is any functional relationship between allergy and plant pathogen defense. PR-proteins are not only induced by pathogen attack, but in a number of cases also by environmental stress (Lamb *et al.*, 1989; Taylor *et al.*, 1990; van de Loeht *et al.*, 1990). Recent investigations showed an even more distinct correlation between the level of *Bet v I* in leaves and pollen and air pollution in regard to NO_x. Pollen and leaves were collected from trees of heavily polluted areas throughout the year and the amount of *Bet v I* determined. In pollen from trees of polluted areas the level of *Bet v I* was significantly higher compared to the amount of *Bet v I* found in pollen from trees in healthy areas. Investigating the amount of *Bet v I* in leaves, the situation differed: *Bet v I* accumulated during the year in the leaves of trees from healthy areas as well as from polluted areas. Only the senescence of leaves seems to correlate with increasing air pollution thus leading to a higher level of *Bet v I* in leaves derived from polluted areas at an earlier time of the year (Breitenbach *et al.*, 1992). Further, more detailed experiments are underway to confirm these results.

Acknowledgements—This work was supported by a grant from the Fonds zur Förderung der wissenschaftlichen Forschung (grant No. S6002-BIO).

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