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High-Level Expression in *Escherichia coli* and Purification of Recombinant Plant Profilins: Comparison of IgE-Binding Capacity and Allergenic Activity

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Because of their structural similarity and ubiquituous distribution as actin binding proteins, plant profilins represent important cross-reactive allergens for almost 20% of patients suffering from Type I allergy to pollen and other plant products. The cDNAs coding for three birch profilin variants (Tyr44, Glu47, and Asn47), timothy grass profilin, and three tobacco profilin isoforms (ntprof1-3) were expressed at high levels in *Escherichia coli* as non-fusion proteins. The recombinant plant profilins were purified to homogeneity by poly (L-proline) affinity chromatography and showed comparable capacity to bind IgE-antibodies from profilin allergic patients. All recombinant plant profilins elicited dose-dependent histamine release from basophils of a profilin allergic patient and induced immediate type skin reactions. It is concluded that profilins from different plant species share IgE-epitopes and allergenic properties. Plant profilins therefore constitute a family of functional pan-allergens which may substitute each other for diagnosis and specific immunotherapy.

Birch profilin (Bet v 2) was discovered as a highly cross-reactive allergen by immunoscreening of a birch pollen cDNA library using IgE-antibodies from an allergic patient (1). Using immunological and proteinchemical techniques, profilins were shown to represent cross-reactive allergens in monocot and other dicot pollens as well as in plant derived food (2-4). Their potential to act as such wide spread cross-reactive allergens could be explained by the fact that profilins act as actin-binding and phosphoinositide-binding proteins in higher plants (5-9). Moreover it was demonstrated that profilin expression is highly upregulated in mature and germinating pollen, indicating that profilin might represent a developmentally upregulated microfilament precursor for pollen germination (10, 11). Although plant and mammalian profilins share a rather low degree of sequence homology it could be demonstrated *in vitro* and *in vivo* that plant and animal profilins bind to actins from different species and can substitute each other (5, 7, 12, 13). This is due to the fact that despite low sequence homology, major structural features of mammalian and plant profilins are conserved as was recently demonstrated by x-ray crystallography (14, 15; Fedorov *et al.*, unpublished data).

In the present study the cDNAs coding for three birch profilin variants (16), timothy grass profilin (17) and three tobacco profilin isoforms (18) were expressed at high levels in *E. coli* and purified to homogeneity. Purified recombinant plant profilins were compared for their ability to bind IgE-antibodies from profilin allergic patients and their biological activity ie. to elicit allergic effector reactions. It is demonstrated that IgE-antibodies crossreacted with the different plant profilins regardless their botanical relation. This supports the concept that

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profilins from different plant species represent a family of cross-reactive allergens and may substitute each other for diagnosis and therapy.

MATERIALS AND METHODS

Biological Materials, Plasmids, and E. coli Strains

Allergic patients were characterized by positive case history, skin prick tests and RAST serology as well as by testing sera for IgE-reactivity with recombinant birch and timothy grass pollen allergens (19, 20). The rabbit antiserum which was raised against a 25 amino acid peptide of the birch profilin C-terminus (RP4) is described (5). Plasmid pET 17b was purchased from Novagen, Maddison, USA and represents a T7 promotor based expression plasmid (21). *E. coli* strain BL21 contained the lysogenic DE3 prophage which harbours the T7 RNA polymerase gene (22).

Expression of Plant Profilins as Non-fusion Proteins

The cDNA coding for timothy grass pollen profilin was PCR amplified from recombinant λ gt 11 phage (17) as template using the following oligonucleotides: P-prof1: 5′ GG*G AAT TC*C ATA TGT CGT GGC AGA CGT AC 3′ (Eco R I: italics; Nde I: underlined) P-prof2: 5′ CGG GGT ACC CTA CTA CAT GCC TTG TTC AAC 3′ (Kpn I: underlined). For the PCR amplification of the tobacco profilin isoforms the oligonucleotide pair: T-prof1: 5′ GG*G AAT TC*A TTA ATA TGT CGT GGC AAA CAT ATG T 3′ (Eco R I: italics; Asn I: underlined) T-prof2: 5′ CGG GGT ACC CTA CTA ATA GCC CTG GTC AAC AAG 3′ (Kpn I: underlined) was used. The cDNA coding for timothy grass profilin was amplified from 10^6 lambda gt11 phage which were boiled to release DNA, whereas the cDNAs coding tobacco profilin isoforms were amplified from 10 ng of the corresponding plasmid DNA (18). The PCR amplification was done according to standard protocols (23) using a GeneAmp PCR Core Reagents kit (Perkin Elmer, Cetus, Emeryville). The PCR product of timothy grass profilin was cut with Nde I/Kpn I and the amplified cDNAs coding for the three tobacco profilin isoforms were cut with AsnI/Kpn I, purified by preparative agarose gel electrophoresis and ligated into the NdeI/KpnI cut plasmid pET 17b. The ligated plasmids were then transformed into *E. coli* Bl21 (DE3) (22). Transformed *E. coli* were then plated onto LB plates containing 100mg/l ampicillin. After colonies became visible, expression of recombinant proteins was induced for 3 hours by overlay with nitrocellulose filters that had been soaked in 10 mM IPTG (isopropyl-β-thiogalactopyranoside). Colonies were disrupted by freeze thawing of the filters and the colonies which expressed recombinant plant profilins were identified by immunoscreening with serum IgE from profilin allergic patients. Clone 163 expressing recombinant timothy grass profilin and clones 31, 13, 10 expressing recombinant tobacco profilin isoforms (ntprof 1-3) were analyzed by DNA restriction analysis and sequencing of the inserted cDNA to exclude PCR introduced mutations (24).

Birch profilin mutants (Tyr44, Glu47, Asn47) were generated by site directed *in vitro* mutagenesis of the wild type birch profilin cDNA using a Chameleon *in vitro* mutagenesis kit (Stratagene, La Jolla, CA) as described (16). *E. coli* BL21 (DE3) containing the plant profilin expression plasmids were grown in 50 ml LB containing 100μg/ml ampicillin to an optical density (OD) 600nm of 0.4 and induced by addition of IPTG to a final concentration of 1mM for 4 hours. Cell pellets were stored at −20°C until protein purification.

Purification of Recombinant Plant Profilins

The cell pellets from 250 ml *E. coli* cultures grown from the plant profilin clones were resuspended in 25 ml 1xPHEM-Tx buffer (25). Cells were homogenized using an ultraturrax (Ika, Germany) and centrifuged at 18.000 rpm at 4°C for 15 minutes to remove insoluble debris. Supernatants were applied to poly (L-proline) columns prepared as described (25, 26). The columns were washed with TBS until the baseline at OD 280nm was reached. Recombinant plant profilins were then eluted with TBS containing 6 M Urea. The eluent fractions were pooled and dialyzed against water. Prior to SDS-PAGE the protein concentrations were determined using the Micro BCA kit (Pierce, Rockford, Illenois). The purity of the recombinant plant profilins was documented by Coomassie Blue staining of SDS-PAGE gels (27).

Antibody Binding to Recombinant Plant Profilins (ELISA, Immunoblot)

Immunoblotting. Approximately 1 μg/cm purified recombinant plant profilins were separated in a 14% preparative SDS-PAGE (28) and blotted onto nitrocellulose (Schleicher & Schuell, Dassel, Germany) (29). Nitrocellulose strips were incubated with 1:10 diluted sera from profilin allergic patients or a rabbit anti-birch profilin C-terminus antiserum (RP 4). Bound IgE was detected with ^{125}I labelled anti-human IgE-antibodies (RAST, Pharmacia, Uppsala, Sweden) and bound rabbit antibodies were detected with a ^{125}I labelled donkey anti-rabbit antiserum (Amersham, Buckinghamshire, UK) as described (2, 5).

Competitive ELISA. Twenty μg/ml purified recombinant birch profilin (30) was coated to ELISA plates (100μl/well) (Nunc, Maxisorb, Roskilde, Denmark). Sera from profilin allergic patients were diluted 1:5 and preincubated

with different concentrations (5μg, 0.5μg, 50ng, 5ng) of each purified recombinant plant profilin before binding was measured as described (31).

Histamine Release Assays

Granulocytes were isolated from heparinized blood samples taken from a profilin allergic individual and an allergic patient without profilin specific IgE by dextran sedimentation (32). The cells were incubated with different concentrations of the purified recombinant plant profilins and several controls (recombinant Bet v 1 (33), histamine release buffer and anti-human IgE antibodies; data not shown). Histamine which was released in the supernatant was measured by radioimmunoassay (Immunotech, Marseille, France). Results were obtained as mean values from triplicate determinations and are expressed as percentage of total histamine release. Total histamine was determined in the supernatants after freeze thawing of the cells.

Skin Prick Testing

Skin prick tests were performed on the allergic patients forearm. Ten μl of each solution was placed at least 3cm apart to avoid false-positive results (34, 35). Recombinant plant profilins were freshly dissolved in 0.9% sterile sodium chloride solution at concentrations of 100μg/ml and 10 μg/ml. As controls, 10 μl each of commercial (SQ standardized quality) birch pollen extract and histamine hydrochloride (ALK, Horsholm, Denmark) were used. Each drop was pricked with a fresh prick lanzette (ALK, Horsholm, Denmark). Wheal and flare reactions were recorded 20 minutes after testing with a ball point pen. The wheal area was transferred by a scotch tape to paper and the results were documented by photography. The mean wheal diameter (Dm) in mm was calculated by measuring the maximal longitudinal diameter (D) and the maximal transversal diameter (d) according to the formula $[D+d]/2 = Dm$.

RESULTS

High-Level Expression of Birch Profilin Mutants, Timothy Grass Profilin and Tobacco Profilin Isoforms in Escherichia coli as Non-fusion Proteins

We expressed recombinant plant profilins at high levels using plasmid pET 17b which was transformed into *E. coli* BL21 (DE3). Recombinant timothy grass profilin, birch profilin variants Glu47, Asn47 and tobacco profilin isoform 3 represented approximately 25% of the soluble protein fraction obtained after freeze thawing of the *E. coli* cells as was estimated by Coomassie blue staining (data not shown). In contrast, much lower amounts of birch profilin Tyr44, tobacco profilin isoforms 2 and 3 (approximately 5% of total *E. coli* proteins) were found in the soluble protein fraction. Most of these profilins were present in the insoluble inclusion body fraction (Fig. 1).

Purification of Recombinant Plant Profilins by Poly(L-proline) Affinity Chromatography

All recombinant plant profilins could be purified from the soluble *E. coli* protein fraction by using poly (L-proline) affinity chromatography in a single step purification protocol. The yields of birch profilin Tyr44 and tobacco profilin isoforms 2 and 3 were lower compared to the other plant profilins which was due to the low degree of solubility and less content of the proteins in the soluble protein fraction. Approximately 1-2 mg recombinant plant profilins were purified from a 250ml *E. coli* culture. Figure 2 shows a Coomassie blue stained gel containing the purified recombinant plant profilins (birch wild type, birch Tyr44, birch Glu47, birch Asn 47, tobacco profilin isoforms 1-3 and timothy grass profilin). More than 95% pure recombinant plant profilins were obtained which migrated at approximately 14 kD. In the case of birch Tyr44 and tobacco profilin 2 (ntprof 2) two bands were stained.

Antibody-Binding Capacity of Recombinant Plant Profilins

Nitrocellulose blotted recombinant plant profilins were probed with serum IgE from 5 profilin allergic patients, serum from a non-allergic individual and a rabbit anti-birch profilin (C-terminus, RP4) antiserum. Nitrocellulose blotted recombinant plant profilins showed comparable IgE-binding capacity (Figure 3) and reacted with the rabbit antiserum which was raised against a peptide comprising the 25 C-terminal amino acids of birch profilin (5). The capacity

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birch            MSWQTYVDEHLMCDIDGQGQQ*LAASAIVGHDGSVWAQSSSFPQFKPQEI    50
birch (Tyr44)    --------------------*----------------------Y-----
birch (Glu47)    --------------------*-----------------------E--
birch (Asn47)    --------------------*-----------------------N--
timothy          -------------E-E-H***H--SA--L----T-----AD------E--
tobacco 1        --------D---A--E--QGHH---A--L-----------T--K---E--
tobacco 2        --------D---A--E--QGNH---A--L-N---------TT--K---E--
tobacco 3        --------D---V--E--*GQH---A--L----------PH--K---E--

birch            TGIMKDFEEPGHLAPTGLHLGGIKYMVIQGEAGAVIRGKKGSGGITIKKT   100
birch (Tyr44)    --------------------------------------------------
birch (Glu47)    --------------------------------------------------
birch (Asn47)    --------------------------------------------------
timothy          -------D---------MFVA-A--------P-R-------A--------
tobacco 1        -N-----D----------F---A--------P-------------------
tobacco 2        -N-----D----------F---A--------P-------------------
tobacco 3        -N-----D---F------F-A----------P-------------------

birch            GQALVFGIYEEPVTPGQCNMVVERLGDYLIDQGL   134
birch (Tyr44)    ----------------------------------
birch (Glu47)    ----------------------------------
birch (Asn47)    ----------------------------------
timothy          -----V---D--M----------------VE--M
tobacco 1        N---I------------------KI----V---Y
tobacco 2        N---I------------------KI----V---Y
tobacco 3        N---I--L---------------KI----V---Y
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FIG. 1. Alignment of the deduced amino acid sequences of plant profilins. Aminoacids which are identical to birch profilin wild type are indicated by dashes. Asterisks indicate gaps introduced for maximal fit.

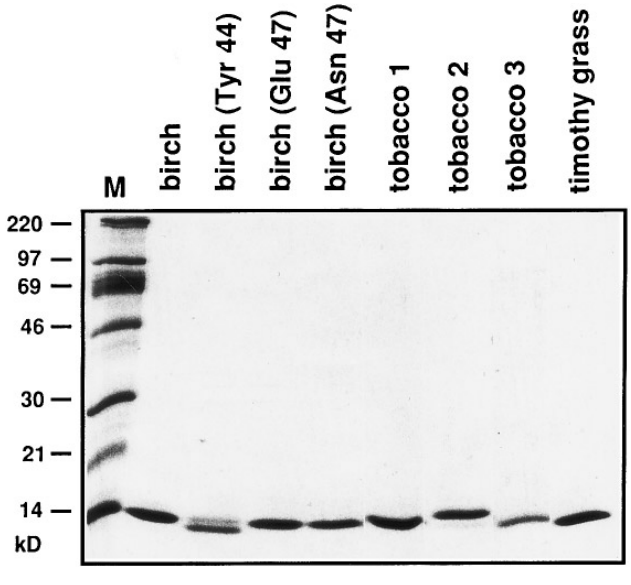

FIG. 2. Coomassie stained SDS-PAGE showing purified recombinant plant profilins. Recombinant plant profilins were purified by poly-(L-proline) affinity chromatography. Approximately 5 μg of each profilin was loaded in each lane of the SDS-PAGE and stained with Coomassie Blue.

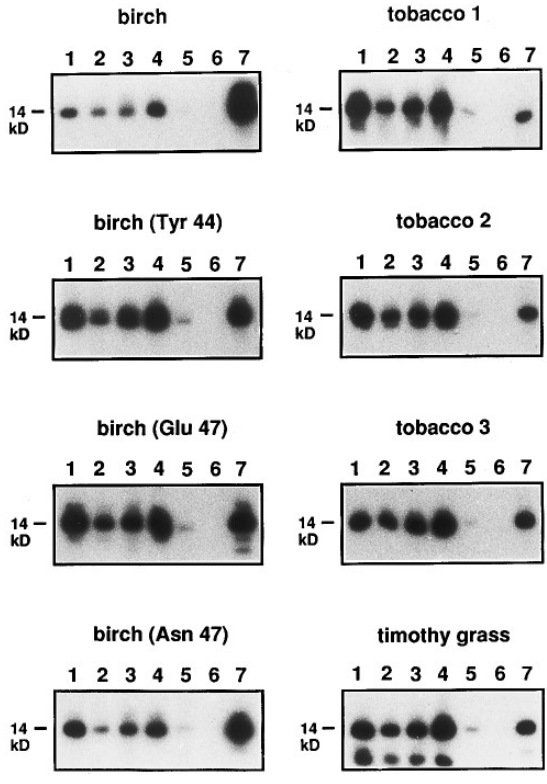

FIG. 3. Antibody binding capacity of recombinant plant profilins determined by immunoblotting. Purified recombinant plant profilins were separated by 14% preparative SDS-PAGE and blotted onto nitrocellulose. Nitrocellulose strips were incubated with serum IgE from 5 profilin allergic individuals (lanes 1-5) and with serum IgE from a non-allergic individual (lane 6). In lane 7, blotted profilins were detected with a rabbit-anti birch C-terminus antiserum (RP4).

of different doses of purified recombinant plant profilins to inhibit IgE-binding to ELISA plate coupled birch profilin (wild type) was investigated by competitive ELISA studies. Sera from three profilin allergic patients were preincubated with 5μg/ml, 500ng/ml, 50ng/ml and 5ng/ml of each of the purified recombinant plant profilins. A comparable and dose dependent inhibition of IgE-binding was observed with all recombinant plant profilins indicating that most IgE epitopes are conserved (Fig. 4).

Purified Recombinant Plant Profilins Induce Dose Dependent Histamine Release from Sensitized Basophils

Basophils from a profilin allergic individual were incubated with different concentrations of purified recombinant plant profilinss. Figure 5 shows that all recombinant plant profilins tested (birch variants, tobacco profilin isoforms, timothy grass profilin) induced dose dependent histamine from basophils of a profilin allergic individual. The optimal concentration for the induction of maximal release was in range between 0.1μg/ml - 1μg/ml for all profilins tested. The spontaneous histamine release measured without addition of allergens was subtracted from the results which are displayed as percentage of total histamine as mean value from triplicate determinations. Basophils from a patient without profilin specific IgE showed no significant histamine release upon incubation with the purified recombinant plant profilins, indicating that the protein preparations were not toxic (data not shown).

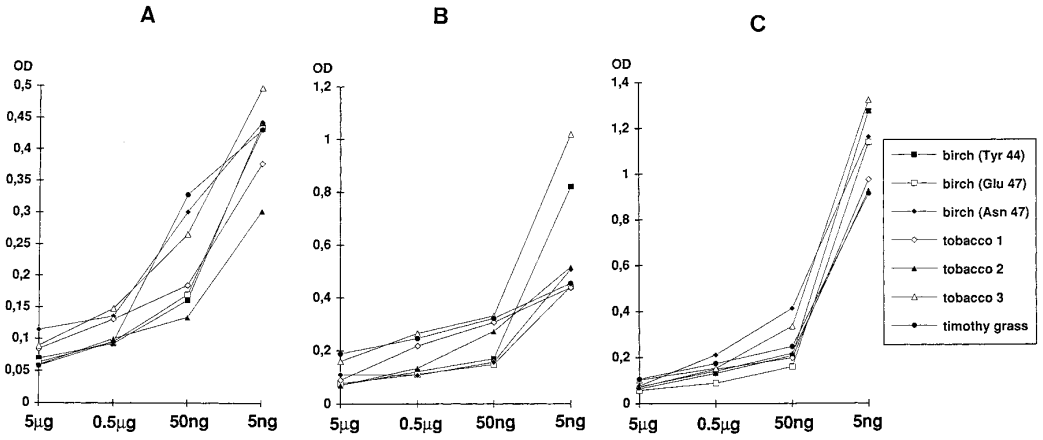

FIG. 4. ELISA competition. Sera from three profilin allergic patients (A, B, C) were preincubated with different concentrations (x-axis: 5μg, 0.5μg, 50ng, 5ng) of recombinant plant profilins (birch profilin variants: birch Tyr 44, Glu 47, Asn 47; tobacco profilin isoforms 1, 2, 3 or timothy grass profilin). IgE binding was measured to ELISA plate coupled recombinant birch profilin and is displayed as optical density (OD) on the y-axis.

Biological Activity of Purified Recombinant Plant Profilins as Determined by Skin Prick Testing

The allergenic activity of the different recombinant plant profilins was compared by skin prick testing of a profilin allergic patient. Different concentrations of the recombinant plant profilins (10 μg/ml, 100 μg/ml) were tested and compared with recombinant birch profilin wild type (30). All birch profilin variants induced comparable wheal reactions to wild type birch profilin. Timothy grass profilin and the tobacco profilin isoform 2 were less anaphylactic at 10 μg/ml whereas similar activity was observed at higher concentrations (100 μg/ml). The wheal reaction which was induced by the recombinant plant profilins was dose dependent and not due to toxic effects because no wheal reactions were observed when a non-allergic control individual was tested. No reactions were observed when the patient was tested with 0.9% sodium chloride which was used to dissolve the recombinant profilins (negative control). Positive reactions were obtained with histamine hydrochloride and commercial birch pollen extract (positive controls) (Table 1).

DISCUSSION

In the present study, the high level expression, purification and immunological characterization of recombinant plant profilins from birch, timothy grass and tobacco pollen is reported. Profilins represent conserved eukaryotic proteins of approximately 14 kD molecular weight with a multitude of different biological functions, comprising binding of actin and phosphoinositides as well as signal transduction (36-38). Plant profilins were in fact discovered as allergens by screening of a birch pollen expression cDNA library with serum IgE from an allergic patient (1). Using immunological and proteinchemical techniques it was demonstrated that profilins represent highly cross-reactive allergens in particular for those patients who suffer from multivalent pollen allergies and Type I allergy to plant derived food (2-4).

To compare the IgE-binding capacity and allergenic activity of profilins from different plants, we have expressed the cDNAs coding for birch profilin variants (16), timothy grass pollen profilin (17) and tobacco pollen profilin isoforms (18) at high levels in *E. coli.* Wild type birch pollen profilin (1, 30) was compared with three birch profilin mutants (Tyr44, Glu47, Asn47) which had been engineered to affect a sequential epitope of a mouse monoclonal

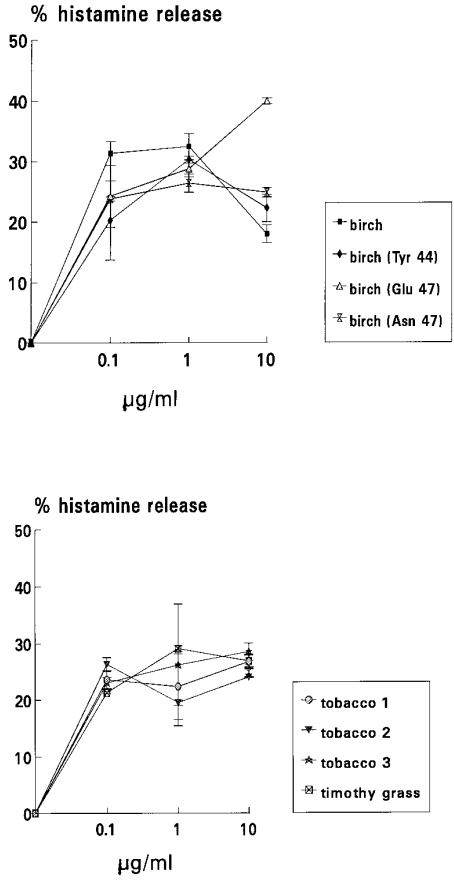

FIG. 5. Induction of dose dependent histamine release from basophils of a profilin allergic patient using purified recombinant plant profilins. Basophil granulocytes from a profilin allergic individual were incubated with different concentrations (0.1, 1, and 10 μg/ml) of recombinant plant profilins as indicated on the x-axis. The upper part of the figure shows the results obtained with the birch profilins and the lower part the results obtained with tobacco profilin isoforms and timothy grass profilin. Percentage histamine release is displayed on the y-axis and the standard deviations are shown for each mesurement.

anti-birch profilin antibody which also bound in an epitopic area for IgE antibodies [Wiedemann *et al.*, submitted; Ball *et al.*, unpublished data]. All recombinant plant profilins were expressed at high levels as non-fusion proteins in *E. coli* and could be purified by single step affinity to poly (L-proline) indicating conservation of their poly (L-proline) binding domains (39, 40). Sera from 5 profilin allergic individuals showed comparable IgE-reactivity to the nitrocellulose blotted recombinant plant profilins. Although all recombinant plant profilins inhibited IgE binding to birch profilin (wild type) completely at concentrations of $>$500ng/ml a slightly different IgE inhibition capacity was noted at low concentrations of inhibitor (5ng/ml). From this it is concluded that the majority of IgE epitopes is shared among the plant profilins, although a minority of some non-crossreactive IgE epitopes are apparently present as well. The allergenic activity of the different recombinant plant profilins was demonstrated by *in vitro* histamine release from basophils of a profilin allergic patient and by skin prick testing. All recombinant plant profilins showed comparable allergenic activity which explains allergic symptoms of profilin allergic individuals upon contact with various pollens and plant products (41). The IgE-binding data and the experiments documenting the biological activity of the

TABLE 1
Skin Prick Activity of Recombinant Plant Profilins

Concentration	10 μg/ml Dm (mm)	100 μg/ml Dm (mm)
Birch	6	11.5
Birch (Tyr 44)	5	10
Birch (Glu 47)	7	13
Birch (Asn 47)	6.5	15.5
Tobacco 1	7.5	7
Tobacco 2	0	7
Tobacco 3	4	10
Timothy grass	0	8
	Amount	Dm (mm)
Birch	10 μl SQ	6
Histamine	10 μl SQ	5.5

plant profilins corroborate earlier findings concerning crossreactivities of profilins and support the concept that profilins from distantly related plants can act as widely spread cross-reactive allergens, which indeed may be designated ''pan-allergens'' (2, 41, 42). As a consequence of these results it is suggested that the different plant profilins can substitute each other for *in vitro* as well as *in vivo* allergy diagnosis. Furthermore it is expected that the different plant profilins might replace each other also for specific hyposensitization treatment and thus facilitate therapy by reducing the number of components needed.

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