

Fra a 1.02 Is the Most Potent Isoform of the Bet v 1-like Allergen in Strawberry Fruit

Katrin Franz-Oberdorf,[†] Bernadette Eberlein,[‡] Kathrin Edelmann,[†] Stephanie Hücherig,[†] Fatma Besbes,[†] Ulf Darsow,[‡] Johannes Ring,[‡] and Wilfried Schwab^{*,†}

[†]Biotechnology of Natural Products, Technische Universität München, Freising, Germany

[‡]Department of Dermatology and Allergy, Technische Universität München, München, Germany

S Supporting Information

ABSTRACT: The strawberry fruit proteins Fra a 1.01E–1.08 are homologues of the major birch pollen allergen Bet v 1. Three of the proteins are known to have essential biological functions in pigment formation during fruit ripening and seem to be responsible for allergic reactions to strawberry fruit. We evaluated the cross-reactive allergenic potential of these putative strawberry allergens in patients allergic to birch pollen. Activation of basophils of eight atopic patients was studied using different concentrations of Fra a 1 isoforms. Bet v 1a was used as control and as atopic patient selection criterion. Although Fra a 1.01E–1.08 have amino acid sequence identities of 74.5–97.5% with Fra a 1.02, the basophil activation mediated by the eight Fra a 1 proteins differed substantially. Fra a 1.03 and Fra a 1.02 showed the highest activation of basophils, 73 and 66% of total basophils, respectively. On the basis of the high relative expression of the gene Fra a 1.02 in ripe strawberry fruits of allergenic varieties, Fra a 1.02 was identified as the main strawberry allergen of the Bet v 1 superfamily. Knowledge of the allergenic potential of Fra a 1.02/1.03 will help to improve food safety and can serve as a valuable marker for the development of red-fruited hypoallergenic strawberry cultivars.

KEYWORDS: allergen, basophil activation test (BAT), Bet v 1-like strawberry allergen, flow cytometry, *Fragaria × ananassa*

INTRODUCTION

Hypersensitivity to strawberry fruit (*Fragaria × ananassa*), due to cross-reactivity with antibodies developed against birch (*Betula verrucosa*) pollen allergens, is commonly reported among the general population, but only a few studies have been reported.^{1–4} The main clinical (in vivo) symptoms caused by birch pollen allergen Bet v 1-mediated cross-reactivity are associated with oral allergy syndromes, local reactions such as itching and dermatitis, and rarely systemic symptoms such as contact urticaria, asthma, and anaphylactic shock.^{5,6} Fra a 1 proteins, homologues of the birch pollen allergen Bet v 1 have been isolated from strawberry fruit,⁷ but also genes homologous to profilins (Fra a 4) and lipid transfer proteins (LTP; Fra a 3) have been identified in a strawberry cDNA library,⁶ implying that proteins of other allergen families are also expressed in strawberry fruit. It is known that in northern Europe sensitization to Bet v 1 is dominant, and it has been reported that about 30% of patients in northern Europe, with self-reported hypersensitivity to food, also show adverse reactions to strawberry.⁷ The strawberry fruit protein Fra a 1 is ranked among subfamily 10 of cytoplasmic pathogenesis-related proteins (PR-10).^{8–10} PR-10 proteins are widely known as part of the plant defense system and are induced as a component of systemic acquired resistance.¹¹

The widespread allergy against birch pollen is often accompanied by adverse reaction to fresh fruits and vegetables due to pollen-allergen specific IgE cross-reactivity to Bet v 1 homologue food allergens.^{12–15} The main reason for this cross-reaction to birch (Bet v 1) is the fact that apple (Mal d 1) and strawberry (Fra a 1) proteins and other members of the PR-10

protein family share almost identical three-dimensional structures.^{12,13} The highly similar structures also imply immunological similarity among the major allergens and consequently analogy of the IgE-binding epitopes on the surface of the target antigens.^{14,15} This is visible in the protein sequence identity between Bet v 1, Mal d 1, and Fra a 1, which ranks from 56.5% (Mal d 1 and Bet v 1) to 58.4% (Bet v 1 and Fra a 1.02) to 71.4% (Mal d 1 and Fra a 1).

In a search for strawberry varieties with low allergen content, a ripe, white-fruited natural mutant, the fruits of which were tolerated by individuals affected by allergy, was found to be virtually free from strawberry Fra a 1 allergen.¹⁶ Proteome analysis of the white-fruited variety and transient RNAi-mediated silencing of *Fra a 1* genes in strawberry fruits of the red-fruited cultivar Elsanta revealed that the expression levels of several catalytically active key enzymes of the anthocyanin biosynthesis pathway and levels of anthocyanins and upstream metabolites were significantly reduced concomitantly with reduced *Fra a 1* expression.^{16–19} These results demonstrated that Fra a 1 proteins have an essential biological function in pigment formation in strawberry fruit.¹⁷

Sequencing of Bet v 1-like genes in the cultivated strawberry revealed only little variability in amino acid sequence (Fra a 1 isoforms A–E).²⁰ However, the recent identification of novel Fra a 1 genes and the genome sequence of the related species

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Table 1. Nomenclature of PR-10 Allergens in *Fragaria × ananassa* and *Fragaria vesca*^{21,22}

gene name <i>Fragaria × ananassa</i>	gene ID ^{22a}	gene name <i>Fragaria vesca</i> ¹⁹	GenBank accession no.	ORF (nt)	length (aa)	pI/MW (kDa)	protein sequence pairwise identity	
							% relating to Fra a 1.02	% relating to Bet v 1
<i>Fra a 1.02</i>	gene07086	<i>Fra v 1.05A</i>	GQ148818	483	161	5.1/17.6	reference	58.4
<i>Fra a 1.01E</i>	gene07080	<i>Fra v 1.10</i>	AM236319	483	161	6.6/17.8	78.9	54
<i>Fra a 1.03</i>	gene07082	<i>Fra v 1.08</i>	GQ148819	480	160	5.6/17.5	80.1	59
<i>Fra a 1.04</i>	gene05122	<i>Fra v 1.06B</i>	KJ507735	483	161	4.9/17.5	95.7	58.4
<i>Fra a 1.05</i>	gene07065	<i>Fra v 1.05B</i>	KJ507736	483	161	5.0/17.5	97.5	59
<i>Fra a 1.06</i>	gene07088	<i>Fra v 1.06A</i>	KJ507737	483	161	4.9/17.5	96.3	57.8
<i>Fra a 1.07</i>	gene05123	<i>Fra v 1.01</i>	KJ507738	483	161	5.6/17.6	74.5	59.6
<i>Fra a 1.08</i>	gene07064	<i>Fra v 1.02</i>	KJ507739	483	161	5.9/17.5	77.0	59

^afvesca v1.1_genemark hybrid version.

Table 2. Clinical and Biological Characteristics of Atopic and Non-atopic Donors

						SPT ^a			
subject	gender	age (years)	clinical reaction to allergens ^a	CAP strawberry (kU/L); ^{a,b}	CAP birch (kU/L) ^{a,c}	strawberry (commercial W/F ^a in mm)	<i>F. vesca</i> (W/F ^a in mm)	<i>F. × ananassa</i> cv. Mara de Bois (W/F ^a in mm)	<i>F. × ananassa</i> cv. Elsanta (W/F ^a in mm)
atopic group									
I	F	52	birch pollen, cat, <i>P. pratense</i>	0.11	71.2	0/0	0/0	2/3	3/9
				class 0	class 5				
II	F	60	birch pollen, alder	0	13.0	0/0	1/1	2/10	4/30
				class 0	class 3				
III	M	34	birch pollen, apple, <i>P. pratense</i> , cat, <i>D. farinae</i>	0.11	45.0	0/0	2/3	0/0	3/3
				class 0	class 4				
IV	F	55	birch pollen, hazel, alder	0.18	51.3	0/0	2/5	6/25	4/25
				class 0	class 4				
V	F	49	birch pollen, apple, <i>P. pratense</i>	6.5	70.9	0/0	1/1	0/0	0/0
				class 3	class 5				
VI	F	24	birch pollen, apple, strawberry, tomato	0.16	16.3	0/0	2/2	3/10	2/2
				class 0	class 3				
VII	F	28	birch pollen, hazel, apple, <i>D. farinae</i>	0.5	>100	0/0	0/1	3/12	3/10
				class 1	class 6				
VIII	F	33	birch pollen, hazel, oak, alder, hazelnut, apple, peach, plum, strawberry	0.01	10.8	0/0	2/3	4/5	3/5
				class 0	class 3				
non-atopic control group									
I	F	32		0.02	0	0/0	0/0	0/0	0/0
				class 0	class 0				
II	F	33		0	0	0/0	0/0	0/0	0/0
				class 0	class 0				

^aSkin prick test (SPT); wheal (W), ≥3 mm positive; flare (F), specific IgE concentration (class 0–6); *P. pratense*, *Phleum pratense*; *D. farinae*, *Dermatophagoides farinae*. ^bStrawberry-specific serum: ImmunoCAP 250, Thermo Fisher Scientific, Phadia GmbH, Freiburg, Germany. ^cBirch pollen-specific serum: IMMULITE 2000DPC, Siemens, Erlangen.

F. vesca show that the genus *Fragaria* contains a family of PR-10 *Fra a 1* homologous genes (<http://supfam.cs.bris.ac.uk/SUPERFAMILY/>).^{16,17,21,22} According to the *F. vesca* genome, it is now clear that *Fra a 1A–1E* represent allelic forms; therefore, we renamed the allelic forms²⁰ *Fra a 1.01A–1.01E*, and the PR-10 family members *Fra a 2* and 3¹⁷ *Fra a 1.02* and *Fra a 1.03*.²³ Although the *Fra a 1.01E* isoform has been described as the major allergen of strawberry fruit,^{16,20} recent qPCR analyses demonstrated that only the *Fra a 1.02* gene shows a ripening-related expression pattern.¹⁷ The highest transcript level was detected in the red ripe fruit, whereas the genes *Fra a 1.01E* and *Fra a 1.03* were barely expressed in this tissue.

The aim of this study was to determine the cross-reactive allergenic potential of recombinant *Fra a 1* proteins by a basophil activation test (BAT) using basophils from birch

pollen allergic patients to identify the main strawberry fruit allergen. The BAT is a highly sensitive and specific in vitro method to detect traces of functionally active food allergens and can be applied for comparison of the allergenic potential of proteins.²⁴ The flow cytometric BAT was applied in this study as a cellular trial for in vitro diagnosis of IgE-mediated reactions.

MATERIALS AND METHODS

RNA Extraction and Cloning of *Fra a 1.01E–1.08*. Total RNA of *F. vesca* red-fruited wild type (red WT; undescribed accession), cv. Yellow Wonder (white fruited), and *F. × ananassa* cv. Elsanta and Mara de Bois (greenhouse Freising, Germany) was isolated, and cDNA synthesis was performed as described.^{25,26} The open reading frame (ORF) sequences of *Fra a 1.01E–1.08* were PCR amplified using strawberry cDNA of *F. × ananassa* cv. Elsanta as template and gene-specific primers (Supporting Information Table S2). PCR

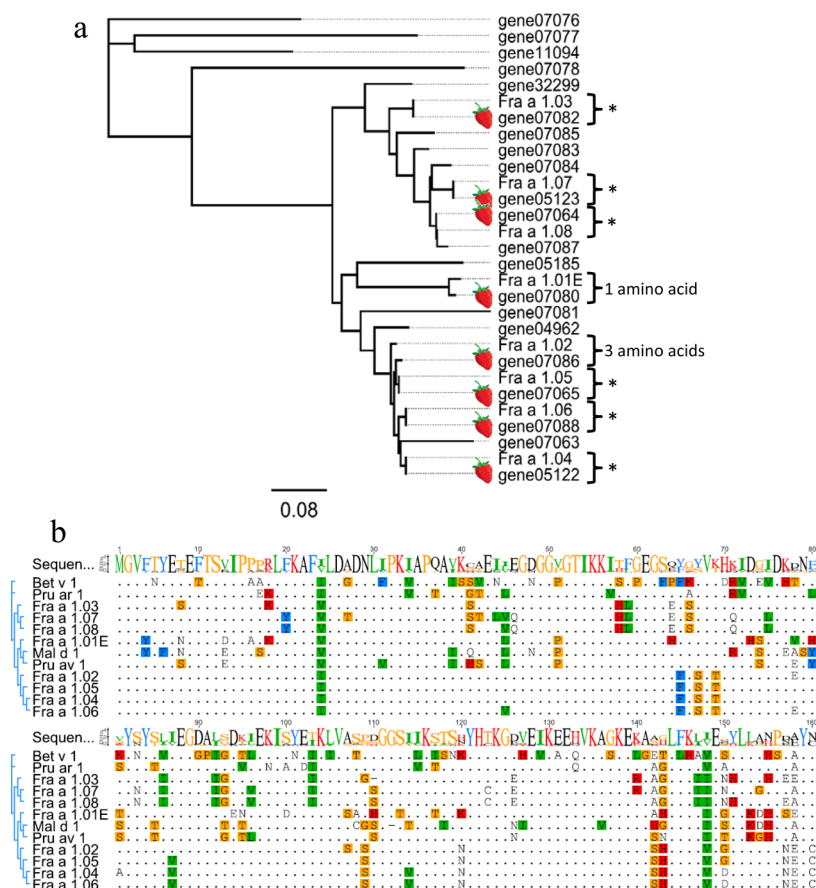


Figure 1. Protein sequence analysis of Fra a 1.01E–1.08. (a) Phylogenetic tree of 21 PR-10 family members of the Bet v 1 superfamily in the *F. vesca* genome (deduced from the genes shown) and proteins analyzed using BAT (Fra a 1.01E–1.08) from *F. × ananassa*. The tree was generated by the Geneious (Pro 5.5.4) Tree Builder (Jukes Cantor genetic distance model and neighbor-joining method). The scale bar indicates the average number of amino acid substitutions per site. The asterisk denotes sequences that are identical in *F. vesca* and *F. × ananassa*; otherwise, the number of amino acid changes is shown. Sequences expressed in strawberry fruit are marked by a strawberry clipart image. (b) Protein sequence alignment of Fra a 1.01E–1.08, Bet v 1a, Mal d 1, Pru av 1 and Pru ar 1 (pairwise identity = 73.3%).

fragments were digested with *Sph*I and *Bam*HI (Fra a 1.02, *Sph*I/*Sph*I; Fra a 1.05, *Sph*I/*Bgl*II) and ligated to the predigested pQE70 vector including a C-terminal His-tag (Qiagen, Hilden, Germany). The validated constructs pQE70-Fra a 1.01E–1.08 were transformed in the *Escherichia coli* strain BL21 (DE3)pLysS (Novagen, Darmstadt, Germany). Sequences were identical to those published (Table 1).²²

Heterologous Expression and Purification of Fra a 1 Proteins. Heterologous expression was performed as described.²⁶ The proteins were purified using Profinity IMAC Resin according to ref 26. The protein concentration was measured as described.²⁷ Purity was evaluated by SDS-PAGE and Coomassie staining, whereas the identity of the purified proteins was confirmed by Western blot using the monoclonal anti-6× His AP antibody (Abcam, Cambridge, UK). EMBL/GenBank accession numbers are listed in Table 1.

Strawberry Protein Extracts for Skin Prick Test. Proteins were extracted from ripe strawberry fruits of different white- and red-fruited genotypes (*F. vesca* red WT, cv. Yellow Wonder, *F. × ananassa* cv. Elsanta and Mara de Bois) using the phenol extraction procedure as described.⁷

Atopic and Non-atopic Groups. Eight atopic patients (seven females, one male, 24–60 years, mean age = 41.9 ± 33 years) with known type I allergy to birch pollen and two healthy non-atopic patients (two females, 32–33 years, mean age = 32.5 ± 2.2 years) were included in the study (Table 2). All atopic patients had a positive skin prick test (SPT) to birch pollen (data not shown) and were without medication against birch pollen allergy for at least 14 days before blood sampling. The control group showed a total IgE level of <0.1 kU/L (data not shown). The BATs were performed outside the birch pollen season, which lasted in Munich from April 4 to May 27, 2013.

The ethics committee of the Technische Universität München approved the study. All subjects were enrolled in the study only after giving written informed consent.

BAT by Flow Cytometry. For quantitative determination of basophil activation induced by Bet v 1a (Biomay, Vienna, Austria), Fra a 1.01E–1.08, and the empty vector (EV) control pQE70 (*E. coli* cells harboring the empty vector were extracted in the same way as *E. coli* expressing the allergens), a commercial kit Flow CAST was applied (Bühlmann Laboratories AG, Schönenbuch, Switzerland) and performed as described.²⁴ The recombinant proteins were used at four different concentrations (500, 50, 5, 0.5 ng/mL). A negative control without allergen, pQE70 EV, and both positive controls (anti-FceRI and fMLP) included in the kit were treated like the samples with allergen. Analysis was performed using a FACScan flow cytometer (Becton-Dickinson Immunocytometry System, Heidelberg, Germany) and CellQuest software. Acquisition was performed on 500 cells for each sample. Results with >15% were regarded as positive.²⁸

Quantitative Real-Time PCR Analysis. QPCR was performed using SensiMix SYBER Hi-ROX (Bioline, Luckenwalde, Germany) and as template cDNA of four different strawberry genotypes (*F. vesca* red WT, cv. Yellow Wonder, *F. × ananassa* cv. Elsanta, Mara de Bois) were used for targeting *Fra a 1.01E/1.02/1.03* and interspacer gene (FaRIB413),²⁹ respectively. The qPCR analysis was performed as described¹⁷ using gene-specific primers (Supporting Information Table S1). Relative gene expression was quantified using the $2^{-\Delta\Delta CT}$ method.³⁰

Statistical Analysis. The box plot was created by using the software R (The R Foundation for Statistical Computing, R version

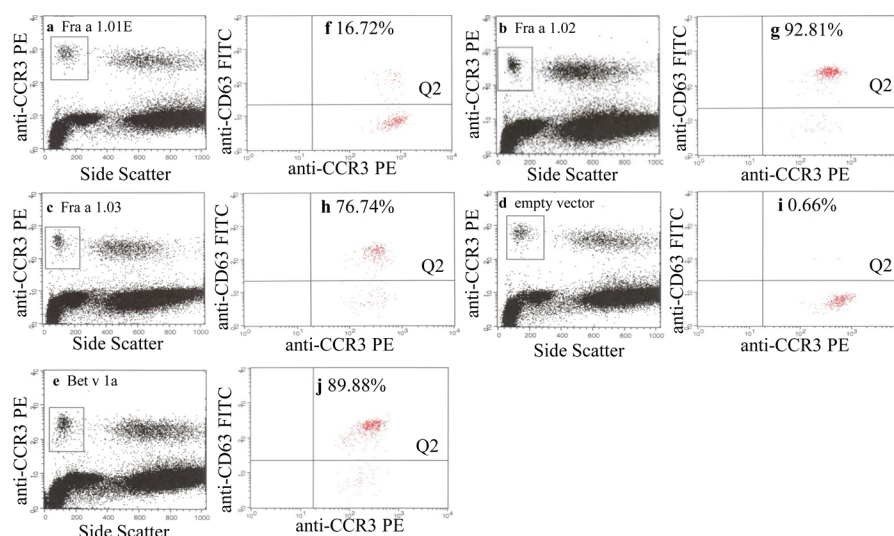


Figure 2. Flow cytometry analysis. Dot plot examples of basophil gating using CCR3 selection marker in basophils from a birch pollen allergic individual after incubation with the putative strawberry allergens Fra a 1.01E (a), Fra a 1.02 (b), Fra a 1.03 (c), the negative control pQE70 EV (d), and the positive control Bet v 1a (e) at a concentration of 50 ng/mL (exemplary selection). Detection of activated basophils by CD63+ expression in anti-CCR3-gated basophils (f–j). Activated basophils (f–j) are expressed as the percentage of CD63+ positive cells in the upper right quadrant (Q2). A result $\geq 15\%$ of CD63+ positive cells was considered as positive.

2.13.0). Statistical significance levels between the variable groups and the control group were calculated using one-way analysis of variance (ANOVA). Differences with $P < 0.05$ were considered significant.

RESULTS

Eight Recombinant Fra a 1 Proteins. The genome of *Fragaria vesca* contains a class of 15–46 genes encoding proteins of the PR-10 subfamily of the Bet v 1 superfamily depending on the selection criterion (<http://supfam.org/SUPERFAMILY/>).^{16,17,21,22} We selected 21 sequences that are most similar to Fra a 1.01E/1.02/1.03 for phylogenetic analysis (Figure 1). Eight of the 21 *F. vesca* genes are expressed in strawberry fruit (data not shown) and were therefore selected for cloning from *F. × ananassa* fruit and further analysis (Table 1).^{17,31–33} Six of the encoded proteins from *F. × ananassa* are identical to those from *F. vesca*, whereas Fra a 1.01E and Fra a 1.02 show one and three amino acid exchanges, respectively, in comparison with their orthologues from *F. vesca*. The eight Fra a 1 protein sequences isolated from *F. × ananassa* cv. Elsanta exhibit 99 identical sites of 160–161 amino acids (61.5%) and a pairwise identity of 73.3% (Figure 1b).³¹ The affinity-purified Fra a 1 proteins were highly pure ($>95\%$) and showed a predicted protein size at about 18 kDa (Table 1; Supporting Information Figure S1).

Characterization of Patient Groups. The eight atopic patients with birch pollen allergy showed partly positive clinical reactions (oral allergy syndrome (OAS), *Rhinoconjunctivitis allergica* (RA)) against apple, tomato, and other related allergen-harboring fruits and vegetables (Table 2). Two patients had a clear-cut history of strawberry allergy. Two of eight patients exhibited strawberry allergen specific IgE, one class 1 and one class 3. However, the results for birch pollen allergen specific IgE concentration ranged from class 3 to 6. None of the atopic test persons showed an allergic reaction by SPT using the commercially available strawberry extract. The SPT was negative in all patients with *F. vesca* red WT (wheal ≤ 3 mm), positive in four cases with *F. × ananassa* cv. Mara de Bois, and positive in six cases with cv. Elsanta. In the control group no allergen-specific IgE, neither to birch pollen nor to

strawberry fruit extract, was found. The two controls did not develop any clinical symptoms against birch pollen and strawberry and showed a negative SPT to birch pollen (data not shown) and the different strawberry extracts (Table 2).

Quality Control of BAT. Basophils of patients included in the present study were clearly identifiable by flow cytometry as they were detected separately from the other blood cells using the selection marker CCR3 and are visible in the left upper square in Figure 2a–e (boxed). Activated basophils tagged with anti-CD63 FITC antibody are located in the upper right quadrant (Q2) of the dot plot (Figure 2f–j). The percentage of CD63+ positive cells represents the amount of activated basophils. They were quantified in the blood samples of all patients after stimulation with Bet v 1a, Fra a 1.01E–1.08 (Table 1), pQE70 EV, and two positive (anti-Fc ϵ RI, fMLP) and a negative control of the kit Flow CAST.

The recombinant strawberry allergens Fra a 1.01E–1.08 displayed a clear dose–response curve with the highest basophil activation upon stimulation with the highest concentration of 500 ng/mL (Supporting Information Figure S2). The positive control, Bet v 1a, reached already a maximum of basophil activation at 0.5 ng/mL, the lowest concentration used in this experiment, and remained at high level. The negative control pQE70 EV showed no basophil activation after stimulation at any concentration (Supporting Information Figure S2). Basophil samples from the control group did not show any up-regulation of CD63+ by Bet v 1a, Fra a 1.01E–1.08 and pQE70 EV (Supporting Information Table S2). However, incubation of basophils from birch pollen allergic patients ($n = 8$), with Bet v 1a, Fra a 1.01E–1.03, and Fra a 1.06–1.08 at a concentration of 50 ng/mL yielded a substantial amount of activated cells in the range from 25 to 73% (Supporting Information Table S2). Fra a 1.04 and Fra a 1.05 did not provoke any basophil activation in the atopic group as the values were identical to those of pQE70 EV (Supporting Information Table S2).

The reliability of the method was confirmed by the analysis of control samples. The mean percentage of CD63+ basophils (Supporting Information Table S2) after incubation with both

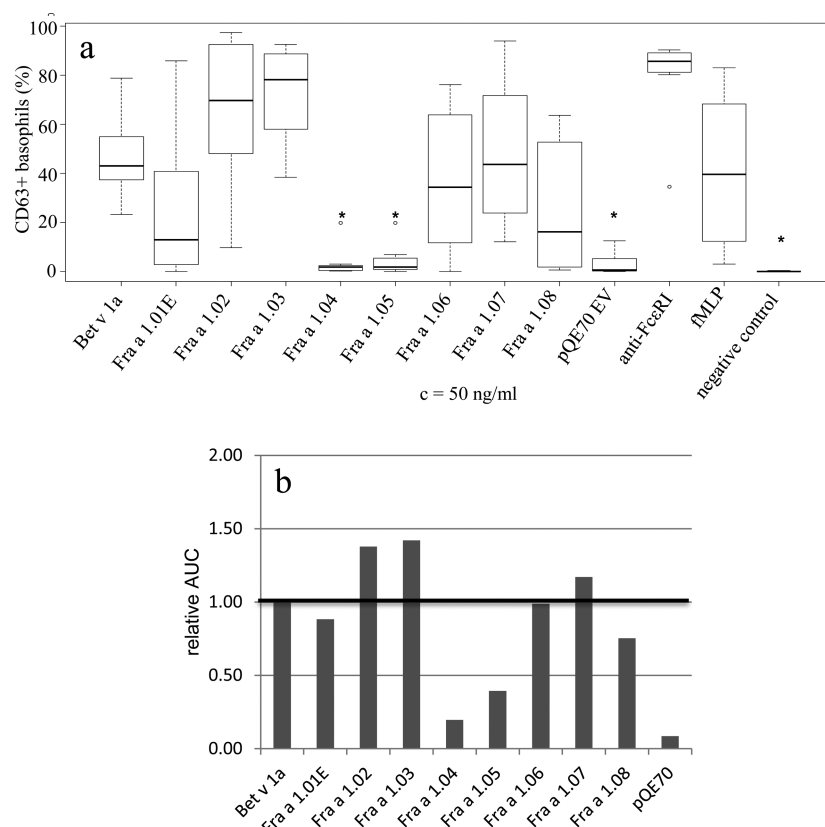


Figure 3. Basophil activation in the BAT with recombinant Fra a 1 proteins. (a) Basophil activation in the BAT in birch pollen allergic ($n = 8$) after incubation with Bet v 1a, pQE70 empty vector (EV), eight Fra a 1 isoforms, two positive controls (anti-FcεRI and fMLP), and the blank value negative control at a concentration of 50 ng/mL. (*) p value < 0.05 referred to Bet v 1a, Fra a 1.02, and Fra a 1.03. (b) Relative area under the curve (AUC) of the activated CD63+ basophil results of all analyzed samples. AUC of the samples are normalized to Bet v 1a.

positive controls of the experimental system anti-FcεRI and fMLP in eight atopic patients was 79.56% ($\pm 0.19\%$) and 40.77% ($\pm 0.30\%$), respectively, compared with average values of 82.82% ($\pm 0.13\%$) and 29.17% ($\pm 0.30\%$) in the control group (Supporting Information Table S2). Similarly, the results of the negative controls (washing buffer and pQE70 EV) in the group of atopic patients differed not significantly from the results in the control group.

BAT of Recombinant Fra a 1 Proteins. A stimulation of the basophils with Bet v 1a, the selective criterion of the patients for this experiment, led to a saturating CD63+ activation with the median at about 40% (Figure 3a). The protein Fra a 1.07 showed a similar median as Bet v 1a but a higher biological variability. The Fra a 1.04 and 1.05 proteins appear to be hypoallergenic isoforms as they had CD63+ basophil activation values of <15%. Three proteins, Fra a 1.06/1.01E/1.08, displayed a low allergenic potential (median around 20–30%) with a high biological variability and activated the basophils in at least four of eight atopic patients. The remaining two proteins, Fra a 1.02 and 1.03, were the most active ones as they activated about 80% of the basophils but also showed a clear biological variability. Every allergic individual exhibited distinct basophil activation after incubation with Fra a 1.03, and seven of eight atopic patients showed a clear activation of basophils after stimulation with Fra a 1.02. Thus, Fra a 1.02 and 1.03 seem to be major allergenic proteins in strawberry fruit. Atopic patients with RA and OAS against strawberry fruit in their allergy history showed, in general, higher values for basophil activation (Table 2).

The calculation of the p values of basophil activation by Bet v 1a, Fra a 1.02, and Fra a 1.03 against all tested recombinant proteins and the controls confirmed that only Fra a 1.04 and 1.05, pQE70 EV, and the negative control show a significant low allergenic potential ($p \leq 0.05$).

Because basophil activation strongly depends on the concentration of the allergens (Supporting Information Figure S2), the relative areas under the curves (AUC, Figure 3b) were calculated as they also take the concentration dependence into account. When AUC of Bet v 1a was set as 1, it became obvious that Fra a 1.04 and 1.05 are hypoallergenic proteins, Fra a 1.01E, 1.06, 1.07, and 1.08 resemble Bet v 1a, and Fra a 1.02 and 1.03 are strong allergens.

Verification of the Major Strawberry Allergen by qPCR Analysis. A quantitative real-time PCR analysis was performed to identify the major Fra a 1 strawberry allergen. The relative expression of both Fra a 1.02 and 1.03 genes was determined as they code for strong allergens (Figure 3a). Transcript levels of Fra a 1.01E were also quantified because the encoded protein has been previously proposed as a major strawberry allergen.^{7,16,20} Fra a 1.01E is primarily expressed in the root, Fra a 1.03 in open flowers, and the Fra a 1.02 gene has the highest relative expression level in the red ripe strawberry fruit.¹⁷ Thus, the relative expression of the target genes Fra a 1.01E/1.02/1.03 was examined in fruits from *F. vesca* red WT and cv. Yellow Wonder and *F. × ananassa* cv. Elsanta and Mara de Bois. The varieties were chosen because the SPT classified fruits from *F. vesca* red WT and cv. Yellow Wonder as

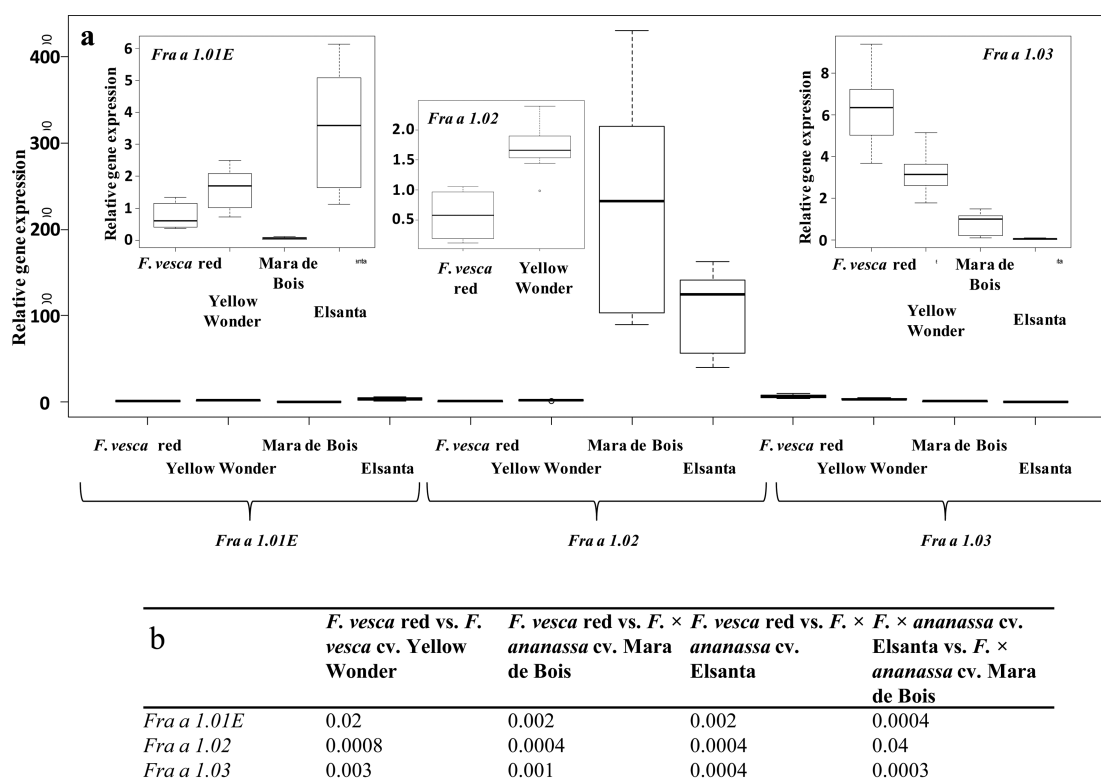


Figure 4. Quantitative RT-PCR analysis of *F. vesca* (red, wild type), *F. vesca* cv. *Yellow Wonder*, and *F. x ananassa* cv. *Mara de Bois* and cv. *Elsanta*. (a) Target genes of the qPCR analysis were *Fra a 1.01E/1.02/1.03*. (Insets) Box plots of low-expressed genes. (b) *p* value of ≤ 0.05 was considered statistically significant.

minor allergenic but fruits from the octoploid *F. x ananassa* cultivars *Elsanta* and *Mara de Bois* as allergenic.

The median of the relative gene expression of *Fra a 1.01E* and *Fra a 1.03* did not exceed a value of 4 or 6, respectively, in the four tested varieties (Figure 4a). Similarly, the relative expression level of *Fra a 1.02* in *F. vesca* red WT and cv. *Yellow Wonder* reached only values of 0.5 and 1.7, respectively. In contrast, the medians of the transcript levels of *Fra a 1.02* in the *F. x ananassa* cultivars *Mara de Bois* and *Elsanta* were nearly 220- and 110-fold higher, respectively, than in both *F. vesca* cultivars. The statistical analysis (Figure 4b) revealed that the relative expression of the three different target genes (*Fra a 1.01E/1.02/1.03*) in *F. vesca* red WT compared with the other three strawberry cultivars as well as between both *F. x ananassa* cultivars *Elsanta* and *Mara de Bois* are statistically significant (*p* values < 0.05). As *Fra a 1.02* is highly expressed in the red ripe fruits of *F. x ananassa* cv. *Elsanta* and *Mara de Bois* (Figure 4a) and allergic individuals exhibit a strong reaction in the SPT against extracts from *F. x ananassa* cv. *Elsanta* and *Mara de Bois* (Table 2), we conclude that the isoform *Fra a 1.02* is the major allergen in strawberry of the PR-10 subfamily of Bet v 1-like proteins.

DISCUSSION

Individuals with birch pollen allergy often suffer from adverse reactions to Rosaceae fruits in central and northern Europe obviously due to the presence of cross-reactive allergens.^{34,35} Structural homologues of the major birch pollen allergen Bet v 1 and other members of the PR-10 protein family have been isolated, for example, from apple (*Mal d 1*) and strawberry fruit (*Fra a 1*).^{11,36} Hence, four and six of eight atopic donors in this study showed a positive SPT reaction against strawberry fruit

extracts from the cultivated genotypes *F. x ananassa* cv. *Mara de Bois* and *Elsanta*, respectively, but wheal and flare reactions were less prominent when an extract from the wild progenitor *F. vesca* was used (Table 2).

The adverse food reactions seen in birch pollen allergic patients are primarily explained by IgE antibodies to Bet v 1, induced by birch pollen, which cross-react with Bet v 1-like proteins in different plant-derived foods. Nearly all birch pollen allergic patients are sensitized to Bet v 1 in the birch tree-rich areas of northern and central Europe, and most of them have Bet v 1-IgE antibodies as the only specificity.³⁴

Allergenic Potential of *Fra a 1* Proteins. We have analyzed eight Bet v 1-like *Fra a 1* proteins of a family of PR-10 members whose genes have been identified in the *F. vesca* genome.²² The eight *Fra a 1* genes are expressed in the fruit tissue, which is consumed fresh or in processed form.³³ Although *Fra a 1.01E* has been proposed as the major strawberry allergen¹⁶ gene expression analyses revealed significantly higher gene expression levels of *Fra a 1.02* in red ripe strawberry fruit (*F. x ananassa*) in comparison with *Fra a 1.01E* and *Fra a 1.03* (Figure 4a). *Fra a 1.03* encodes a second allergenic isoform, besides *Fra a 1.02*.

The eight *Fra a 1* proteins have high sequence similarities between 74.5 and 97.5% with *Fra a 1.02* and between 54 and 59.6% with Bet v 1a (Table 1; Figure 1). Nevertheless, the basophil activation of birch pollen allergic patients differed substantially between these isoforms. *Fra a 1.01E* and *Fra a 1.04–1.08* showed low capacity to activate basophils, probably due to reduced IgE binding.³⁷ Differences in IgE-binding activities are a common attribute within the PR-10 family, despite the high sequence similarity of the members. Some of the structurally, closely related proteins are potent

allergens, whereas others are less allergenic or even non-allergenic. Recently, crystal structure analyses of Bet v 1 variants in complex with numerous ligands identified Phe31 (Figure 1b) as an important amino acid that discriminates high and low IgE binding Bet v 1 isoforms and also induces a drastic change in the binding mode of the model ligand deoxycholate.³⁸ Both allergenic and hypoallergenic isoforms of Fra a 1 contain isoleucine in this position, which implies that this observation is restricted to Bet v 1 isoforms (Figure 1b).

There is a remarkably high sequence similarity between Fra a 1.02 and Fra a 1.04/1.05/1.06 with values of 95.7, 97.5, and 96.3% and only six, three, and five amino acids are replaced, respectively (Figure 1b). Two amino acid exchanges at Ile87Val and Ser107Ala are present in Fra a 1.04, 1.05, and 1.06 compared with Fra a 1.02. The amino acids seem to be important for IgE binding as only Fra a 1.02 and 1.03 are potent allergens. Additionally, Fra a 1.04 and 1.05 share a third amino acid replacement of Glu78Asp, and Fra a 1.04 and 1.06 have in common the amino acid exchanges of Ile114Val and Gly150Asp in comparison with Fra a 1.02. It is assumed that allergens must have at least two distinct and spatially separated antibody binding epitopes to aggregate the IgE complexes, function as allergens, and activate basophils.^{39,40} Recently, antibodies were raised against Bet v 1-derived peptides and were used to compete with human IgE binding to Bet v 1. Two peptides comprising amino acids 30–59 (P2) and amino acids 74–104 (P6) of the Bet v 1 sequence strongly inhibited the binding of patients' IgE to Bet v 1.⁴⁰ It was assumed that P2 and P6 define a surface patch on the Bet v 1 allergen required for efficient basophil activation. Because allergenic Fra a 1.02 and the hypoallergenic Fra a 1.04/1.05 have identical P2 sequences, this putative binding site cannot explain the contrasting immunological effect. However, the two amino acid exchanges Ile87Val and Glu78Asp that differentiate Fra a 1.02 from Fra a 1.04 and 1.05 are localized in the P6 motif. We also propose multiple binding epitopes for Fra a 1 proteins as there are no single amino acid exchanges that clearly differentiate all hypoallergenic from the allergenic isoforms. Thus, IgE peptide mapping comparisons and structural studies of the recombinant Fra a 1 proteins could be useful in future studies to clarify binding of IgE to the strawberry proteins and explain cross-reactivity.

Major Strawberry Allergen. The transcript abundance of *Fra a 1.02/1.03* (encoding allergenic proteins) and *Fra a 1.01E* (encoding the proposed major allergen)²⁰ was determined in fruits of the varieties *F. vesca* red-fruited WT and cv. Yellow Wonder and *F. × ananassa* cv. Elsanta and Mara de Bois. As the *Fra a 1.02* gene is highly expressed in allergenic fruits, Fra a 1.02 was unambiguously identified as the prominent Bet v 1-like allergen in *F. × ananassa* cv. Elsanta and Mara de Bois, the two genotypes that showed the highest stimulation index values in SPT (Table 2). *Fra a 1.01E* and 1.03 were only poorly expressed in the fruit tissues. *Fra a 1.01E* is highly expressed in the root and Fra a 1.03 in open flowers, but overall their relative expression levels are low.¹⁷

Our results clearly demonstrate that small changes in the amino acid sequence can significantly alter the allergenic characteristics of paralogous proteins, although their three-dimensional structures are almost identical. The data also support the role of the ripening-related expression of Fra a 1.02 as the major Bet v 1-like allergen in strawberry fruit. The data are essential for the breeding of hypoallergenic strawberry cultivars using Fra a 1.02 as marker.

■ ASSOCIATED CONTENT

§ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b00488.

Table S1, Primer sequences for cloning of *Fra a 1.01E–1.08* and qPCR analysis of the target genes *Fra a 1.01E/1.02/1.03*; Table S2, CD63+ basophil activation results of atopic and non-atopic donors by eight recombinant Fra a 1 proteins, Bet v 1a and the pQE70 empty vector, two positive controls, and a blank value; Figure S1, SDS-PAGE and Western Blot analysis of the eight recombinant Fra a 1 proteins (10 µg); Figure S2, dose–response data for CD63+ expression in basophils of birch pollen allergic patients after incubation with different concentrations of Bet v 1a, eight recombinant Fra a 1 proteins, and the pQE70 empty vector (EV) (PDF)

Accession Codes

Nucleotide sequence data reported are available in the GenBank databases under the accession numbers provided in Table 1.

■ AUTHOR INFORMATION

Corresponding Author

*(W.S.) Mail: Biotechnology of Natural Products, Technische Universität München, Liesel-Beckmann-Str. 1, 85354 Freising, Germany. Phone: +49(0)1606005463. Fax: +49(0)816171-2950. E-mail: wilfried.schwab@tum.de.

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Notes

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■ ABBREVIATIONS USED

Fra a 1.01E–1.08, *Fragaria × ananassa* 1.01E–1.08; BAT, basophil activation test; LTP, lipid transfer protein; SPT, skin prick test; RA, *Rhinoconjunctivitis allergica*; OAS, oral allergy syndrome; anti-CCR3-PE, monoclonal antibody to human chemokine receptor 3 labeled with phycoerythrin; anti-CD63-FITC, monoclonal antibody to human cluster of differentiation 63 labeled with fluorescein isothiocyanate; anti-FcεRI, highly specific monoclonal antibody binding to the high affinity IgE binding receptor; fMLP, *N*-formylmethionyl-leucyl-phenyl-alanine, unspecific cell activator

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Supporting information

Fra a 1.02 is the major Bet v 1 homologous strawberry allergen

Katrin Franz-Oberdorf¹, Bernadette Eberlein², Kathrin Edelmann¹, Stephanie Hücherig¹, Fatma Besbes¹, Ulf Darsow², Johannes Ring², Wilfried Schwab¹

¹Biotechnology of Natural Products, Technische Universität München, Freising, Germany

²Department of Dermatology and Allergy Biederstein, Technische Universität München, München, Germany

Table S1. Primer sequences for cloning of *Fra a 1.01E-1.08* and qPCR analysis of the target genes *Fra a 1.01E/1.02/1.03*

Table S2. CD63+ basophil activation results

Figure S1. SDS-PAGE and Western Blot analysis

Figure S2. Dose-response data for CD63+ expression in basophils

Table S1. Primer sequences for cloning of *Fra a 1.01E-1.08* and qPCR analysis of the target genes *Fra a 1.01E/1.02/1.03*.

Primer name	Primer sequence (5' to 3')
Fraa1_SphI_fw	GATCGCATGCTGATGGGTGTTTACACTTATGAAAACGAG
Fraa1_BamHI_rv	GATCGGATCCGTTGTATTTCGCTGGGGTGGTCCTTGAG
Fraa2_SphI_fw	GATCGCATGCTGATGGGTGTGTTCACCTTATGAAACCGAG
Fraa2_SphI_rv	GATCGCATGCAACAGTATTCATTAGGATTGGCCAAGAG
Fraa3_SphI_fw	GATCGCATGCTGATGGGTGTGTTCACATACGAATCCGAG
Fraa3_BamHI_rv	GATCGGATCCGTTGTATTCCTCAGGATGGGCCAAGAGG
Fraa4_SphI_fw	GATCGCATGCTGGGTGTCTTCACATATG
Fraa4_BamHI_rv	GATCGGATCCACAGTATTCATTAGGATT
Fraa5_SphI_fw	GATCGCATGCTGGGTGTATTCACCTTATG
Fraa5_BglII_rv	GATCAGATCTACAGTATTCATTAGGATT
Fraa6_SphI_fw	GATCGCATGCTGGGTGTGTTCACCTTATG
Fraa6_BamHI_rv	GATCGGATCCACAGTATTCATTAGGATT
Fraa7_SphI_fw	GATCGCATGCTGGGCGTGTTCACCTTAT
Fraa7_BamHI_rv	GATCGGATCCGTTGTATGCATCAGGGTT
Fraa8_SphI_fw	GATCGCATGCTGGGTGTGTTCACATAT
Fraa8_BamHI_rv	GATCGGATCCGTTGTAGGCCTCAGGGTT
Fra a 1fw	TCCTTATTCCCTGCTTGCTT
Fra a 1rv	AGGTGATCTTCTTGATGGTTC C
Fra a 2 fw	CGTGGAGATCAAGGAAGAGC
Fra a 2 rv	GGAACATCAGCGGAACAAAT
Fra a 3 fw	GTTAAGGCCGGAAGGAAAG
Fra a 3 rv	GATCGAGCAGATTGACACCA

Table S2. CD63+ basophil activation results of atopic (n=8) and nonatopic (n=2) donors by eight recombinant Fra a 1 proteins, Bet v 1a and the pQE70 empty vector (EV; c=50 ng/ml), two positive controls and a blank value.

Stimulus	CD63+ mean [%] atopic group	SD [%] atopic group	CD63+ mean [%] nonatopic group	SD [%] nonatopic group
Fra a 1.01E	24.91	0.30	0.0	0.0
Fra a 1.02	65.89	0.30	0.65	0.003
Fra a 1.03	72.59	0.21	0.55	0.002
Fra a 1.04	3.75	0.07	0.11	0.0008
Fra a 1.05	4.56	0.07	0.13	0.002
Fra a 1.06	36.93	0.29	0.0	0.0
Fra a 1.07	48.08	0.30	0.11	0.002
Fra a 1.08	25.71	0.27	0.20	0.003
pQE70 EV	3.12	0.05	0.48	0.001
Bet v 1a	46.59	0.17	0.21	0.003
FcεRI	79.56	0.19	82.82	0.13
fMLP	40.77	0.30	29.17	0.30
blank value	0.11	0.002	0.31	0.004

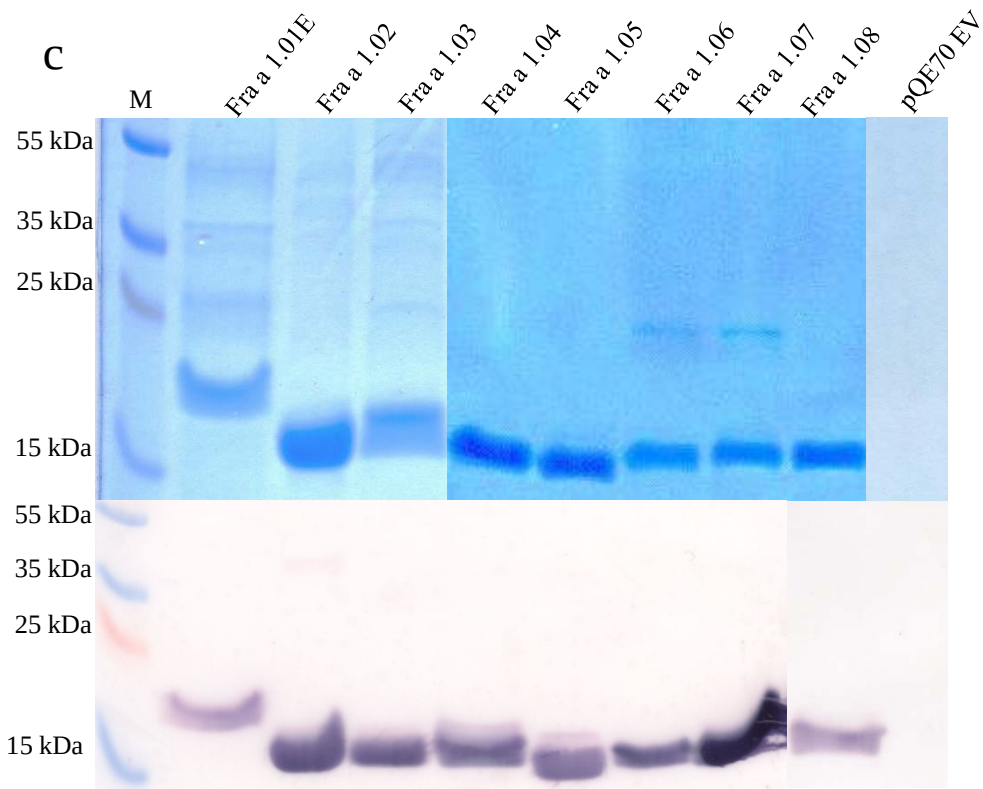


Figure S1. SDS-PAGE and Western Blot analysis of the eight recombinant Fra a 1 proteins (10 μ g). The SDS-PAGE was visualized by Coomassie Brilliant Blue G250. The recombinant proteins in the Western Blot were detected using the monoclonal anti-6 $\times$ His AP antibody. The Fra a 1 isoforms are plotted according their numbers from one to eight. As negative control the empty vector pQE70 (EV) was analyzed. M: protein marker.

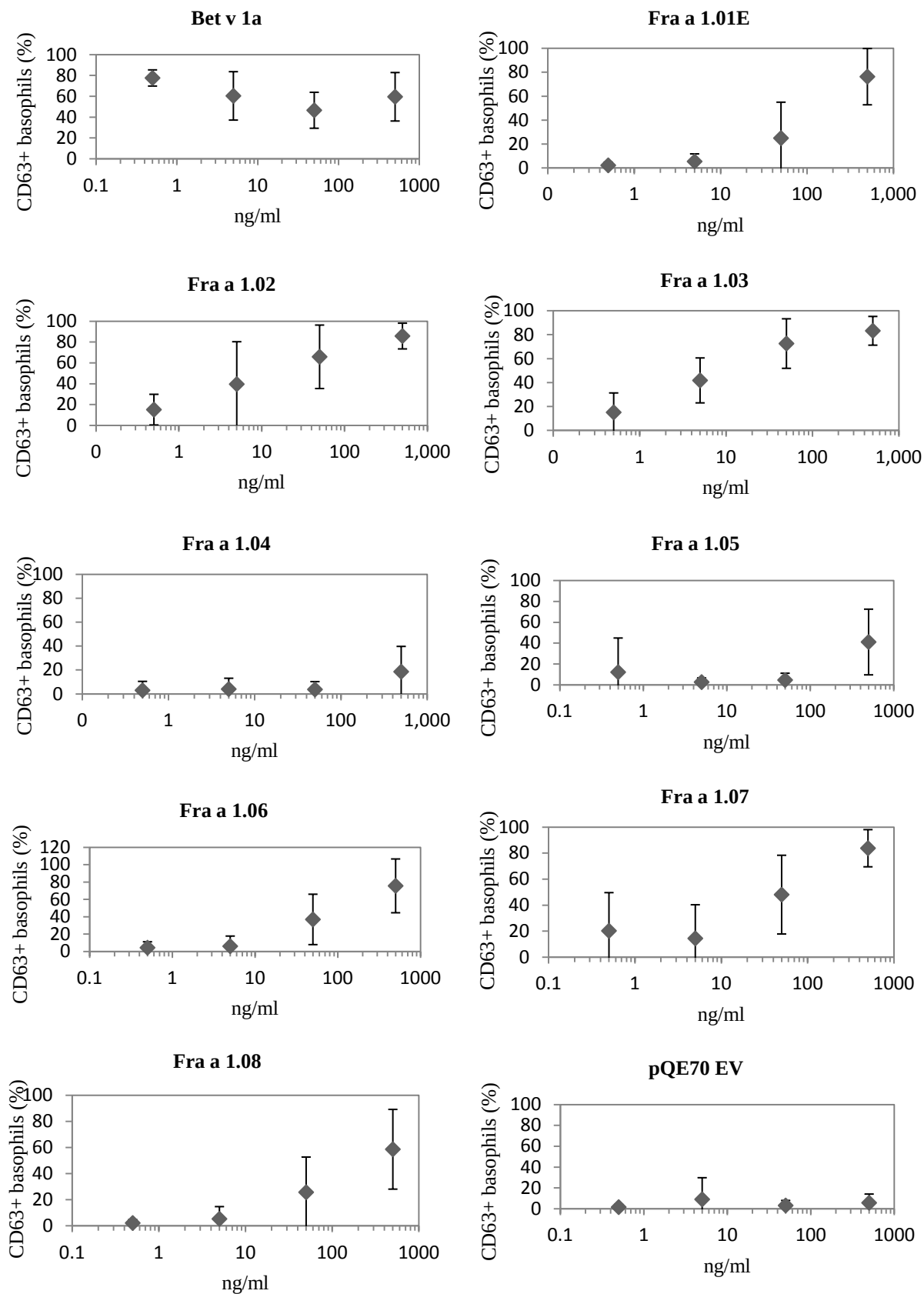


Figure S2. Dose-response data for CD63+ expression in basophils (means $\pm$ SD) of birch pollen allergic patients (n = 8) after incubation with different concentrations of Bet v 1a, eight recombinant Fra a 1 proteins and the pQE70 empty vector (EV). Graphics are represented in log scale.