



White-fruited strawberry genotypes are not *per se* hypoallergenic

Katrin Franz-Oberdorf^a, Bernadette Eberlein^b, Kathrin Edelmann^a, Philip Bleicher^a, Elisabeth Kurze^a, Dominic Helm^c, Klaus Olbricht^d, Ulf Darsow^b, Johannes Ring^b, Wilfried Schwab^{a,*}

^a Biotechnology of Natural Products, Technische Universität München, Liesel-Beckmann-Str. 1, 85354 Freising, Germany

^b Department of Dermatology and Allergy Biederstein, Technische Universität München, Biedersteiner Str. 29, 80802 München, Germany

^c Chair of Proteomics and Bioanalytics, Technische Universität München, Emil-Erlenmeyer-Forum 5, 85354 Freising, Germany

^d The Strawberry Breeding Company Hansabred GmbH & Co. KG, Radeburger Landstr. 12, 01108 Dresden, Germany

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ABSTRACT

The strawberry fruit Fra a 1-proteins are homologues of the major birch pollen allergen Bet v 1 and have essential biological functions in pigment formation during fruit ripening. Patients affected by allergy against birch pollen tolerated fruits of a naturally occurring white-fruited *F. × ananassa* genotype, which showed reduced levels of Fra a 1 proteins along with enzymes of the anthocyanin pigment pathway. We evaluated the cross-reactive allergenic potential of a number of naturally occurring white- and red-fruited strawberry varieties to detect genotypes with low allergenic reactivity, whose fruit might be tolerated by patients with mild allergy. Protein extracts of 51 different strawberry varieties (*Fragaria* × *ananassa*, *F. vesca*, and *F. nilgerensis*) were screened by Western blot analysis with a polyclonal Fra a 1.02 antibody. Besides, activation of basophils of eight atopic patients allergic to birch pollen were studied using Bet v 1a and different concentrations of 15 selected strawberry protein extracts out of the 51 strawberry genotypes. Median percentages of activated basophils stimulated by extracts from white- and red-fruited genotypes ranged from 36 to 84% and 44 to 76%, respectively indicating that white-fruited strawberry are not *per se* hypoallergenic. Protein extracts from white-fruited *F. vesca* cv. Yellow Wonder showed the lowest cross-reactivity but high biological variability. The knowledge about the allergenic potential of different strawberry genotypes may help to improve food safety and can serve as starting point for the development of red-fruited hypoallergenic strawberry cultivars.

1. Introduction

Individuals affected by allergy to strawberry fruit (*Fragaria* × *ananassa*) mostly suffer from the hypersensitive allergic reactions caused by the Fra a 1 allergens in Northern Europe (Franz-Oberdorf et al., 2016). The hypersensitivity is commonly reported among the general population, but only a few studies investigating allergic patients and the allergenic potential of different strawberry varieties has been published (Eriksson et al., 2004; Grattan & Harman, 1985; Hjernø et al., 2006; Kaiser et al., 2016; Taylor, Lever, & Lawrence, 1996; Weltfriend, Kwangstuth, & Maibach, 1995). Fra a 1 proteins, homologues of the birch (*Betula verrucosa*) pollen allergen Bet v 1 lead, due to Bet v 1-mediated cross-reactivity, to the same *in vivo* symptoms like the oral allergy syndrome (OAS),

including itching, dermatitis, and urticaria (Franz-Oberdorf et al., 2016; Kaiser et al., 2016; Karlsson et al., 2004; Zuidmeer et al., 2006). Eight Fra a 1 proteins have been reported from strawberry fruit (Franz-Oberdorf et al., 2016; Karlsson et al., 2004) but in a strawberry cDNA library also genes encoding other putative allergens such as profilins (Fra a 4) and lipid transfer proteins (LTP; Fra a 3) have been identified (Zuidmeer et al., 2006). The strawberry fruit protein family Fra a 1 is ranked among subfamily 10 of cytoplasmic pathogenesis-related proteins (PR-10) (Radauer & Breiteneder, 2007; Rashid, Smith, Nambiar, Frew, & Tarzi, 2011; Schenk et al., 2009). PR-10 proteins are widely known as a part of the plant defence system and are induced as component of systemic acquired resistance (Laimer & Maghuly, 2010; Lebel, Schellenbaum, Walter, & Maillot, 2010). It appears that PR-10 genes constitute a multigene family in which some members are constitutively

Abbreviations: Fra a 1, *Fragaria* × *ananassa* allergen class 1; 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; ITP, immuno tissue printing

* Corresponding author.

E-mail address: wilfried.schwab@tum.de (W. Schwab).

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expressed in defined plant tissues while others are induced under certain environmental conditions (Hyun & Kim, 2011; Lebel et al., 2010). The expression of PR-10 proteins are highly increased as a response of pathogen infection by fungi (Somssich, Schmelzer, Bollmann, & Hahlbrock, 1986; Walter, Liu, Grand, Lamb, & Hess, 1990), bacteria (Somssich et al., 1986), and viruses, as well as by wounding (Warner, Scott, & Draper, 1992) and further adverse environmental conditions (Breiteneder & Radauer, 2004; Hoffmann-Sommergruber, 2002; Laimer & Maghuly, 2010). Therefore, Bet v 1-homologous proteins such as Fra a 1 and consequently the allergenicity of strawberry fruit might be affected and induced by unfavorable biotic and abiotic factors. It is a fact that the sensitization to Bet v 1 in Northern Europe is predominant. Additionally, 30% of patients with self-reported hypersensitivity to food in Northern Europe also report about allergic reactions against strawberries (Karlsson et al., 2004; Radauer & Breiteneder, 2007).

The widespread allergy against birch pollen co-occurs often with adverse reaction to raw food, due to pollen-allergen specific IgE cross-reactivity to Bet v 1-homologue food allergens (Neudecker et al., 2003). Nearly 70% of birch pollen allergic patients show this IgE-mediated food allergy against raw fruits like peach (*Prunus persica*), apple (*Malus domestica*), strawberry (*F. × ananassa*) and some vegetables (Kaiser et al., 2016). The main reason for this cross-reaction between birch (Bet v 1), apple (Mal d 1), strawberry (Fra a 1) and other members of the PR-10 protein family is the identical three-dimensional structure and thus immunological similarity among the major allergens and consequently the analogy of the IgE-binding epitopes on the surface of the target antigens (Gajhede et al., 1996; Neudecker et al., 2003).

In a search for strawberry genotypes with low allergen content, the proteomic variation within and between different varieties has been determined (Alm, Ekefjård, Krogh, Häkkinen, & Emanuelsson, 2007). Multiple proteins, homologous to Bet v 1 were detected. The total allergen content varied due to growth conditions and was lower in a colourless (white-fruited) strawberry variety than in red-fruited genotypes. However only three varieties were tested and the authors supposed that the white-fruited strawberry varieties might have the same ancestry. Thus, it was not clear if a low Fra a 1 content is a general property of white-fruited strawberry genotypes. The ripe colourless fruits were tolerated by individuals affected by allergy and were found to be nearly free from the strawberry allergen (Hjerno et al., 2006). Proteome analysis also revealed that several catalytically active proteins were reduced. These enzymes belong to the anthocyanin biosynthesis pathway, which produces the strawberry fruit pigments including pelargonidin-3-O-glucoside (Griesser et al., 2008; Hjerno et al., 2006; Muñoz et al., 2010).

Next, the Fra a 1 gene was transiently silenced by RNAi in strawberry fruits of red-fruited *F. × ananassa* cv. Elsanta (Hoffmann, Kalinowski, & Schwab, 2006; Muñoz et al., 2010). As a consequence of reduced levels of Fra a 1 mRNAs, fruits were obtained that produced significantly lower levels of anthocyanins and upstream metabolites. This effect is consistent with the down-regulation of the transcript levels of some genes encoding catalytically active enzymes involved in the formation of anthocyanin pigments. These results demonstrate that Fra a 1 proteins have an essential biological function in pigment formation in strawberry fruit (Muñoz et al., 2010). Fra a 1 proteins might be involved in the transport of anthocyanin pathway intermediates mediating the transfer of the metabolites from one enzyme to another. Affinity binding assays between Fra a 1 proteins and flavonoid pathway intermediates like quercetin-3-O-glucuronide, myricetin and (+) catechin support this hypothesis as dissociation constants (K_d) in low micromolar ranges were determined (Casañal et al., 2013; Muñoz et al., 2010).

At least eight Fra a 1 proteins are expressed in the strawberry fruit (Fra a 1.01–1.08) and exhibit substantially different allergenic potentials, although their three-dimensional structures are almost identical (Franz et al., 2013; Franz-Oberdorf et al., 2016). Due to the fruit-

ripening related expression profile of the corresponding gene and its high potential to activate basophils Fra a 1.02 seems to be the major Bet v 1-like strawberry allergen in strawberry fruit (Franz-Oberdorf et al., 2016).

The genus *Fragaria* in the family of *Rosaceae* provides biological and genetic diversity in terms of red-, yellow- and white-fruited, big to small sized, highly aromatic and delicious and unenjoyable varieties. Some species are well known like the small and aromatic woodland strawberry *F. vesca* L. ($2n = 2 \times$) and the large pleasant flavoured garden strawberry *F. × ananassa* Duch. ($2n = 8 \times$) in their countless accessions and cultivars (Ulrich, Komes, Olbricht, & Hoberg, 2007). In 2010 the small genome (240 Mb) of the white-fruited *F. vesca* accession 'Hawaii 4' was fully sequenced and offers now new opportunities for research on the popular genus *Fragaria* (Shulaev et al., 2011). Yet another accession of *F. vesca*, the inbred line Yellow Wonder attracted scientific attention as a possible sequencing candidate and subject of investigation because of the yellowish fruit colour (c) locus in the genome (Deng & Davis, 2001; Slovin, Schmitt, & Folta, 2007). Another mostly unknown *Fragaria* species is the white- and small fruited Asian wild strawberry *F. nilgerrensis* ($2n = 2 \times$) (Noguchi, Mochizuki, & Sone, 1997). The flavour of *F. nilgerrensis* is characterised by its strong fruity and exotic banana-like or apricot/peach-like aroma notes (Ulrich et al., 2007).

The genetic secrets behind the different colours, sizes and flavours of the strawberry species are largely unknown. Impaired expression or mutation of structural genes, e.g. encoding flavanone-3-hydroxylase in the case of *F. vesca* cv. Yellow Wonder (Deng & Davis, 2001), and anthocyanidin glucosyltransferase in the case of *F. chiloensis* subsp. *chiloensis* (Griesser et al., 2008; Griesser et al., 2008; Haertl et al., 2017; Salvatierra, Pimentel, Moya-Leon, Caligari, & Herrera, 2010; Saud et al., 2009), or regulatory factors such as *FaMYB10* (Hawkins, Caruana, Schiksnis, & Liu, 2016; Medina-Puche et al., 2014) are hold responsible for the loss of colour (Griesser et al., 2008; Hjerno et al., 2006; Muñoz et al., 2010).

Strawberries, as the most popular berries in Europe exhibit a high nutritional and phytochemical content comprising among others vitamins, anthocyanins, ellagitannins, quercetin, and catechin (Afrin et al., 2016). Some clinical studies, *in vivo* animal and *in vitro* cell culture studies show clear healthy effects of strawberry polyphenols in postprandial inflammation, insulin sensitivity and more. In a time of an alarming increase of chronic diseases like cardiovascular disease, cancer, Alzheimer's disease and diabetes the anti-inflammatory, anti-carcinogenic, antiproliferative, antioxidant, antihyperglycemic, anti-obesity, antimicrobial, neurodegenerative and cardioprotective activities of strawberry polyphenols offer a healthy effect (Afrin et al., 2016; Edirisinghe et al., 2011; Forbes-Hernandez et al., 2016; Huang, Park, Edirisinghe, & Burton-Freeman, 2016). The role of a glycemic control of strawberry polyphenols can be explained by enhancing the insulin production, promoting the proliferation of pancreatic β -cells and by regulating glucose metabolism using various mechanisms *via* modification of oxidative stress, inflammation or perceived energy status of the cells (Coleman, Hurst, Sawyer, & Kruger, 2016). The antioxidant properties of strawberry polyphenols and the healthy effects are proven and everyone should benefit from this anti-inflammatory, anti-carcinogenic and antiproliferative effects by simply include strawberries in the daily diet (Edirisinghe & Burton-Freeman, 2016; Forbes-Hernandez et al., 2016). Considering that strawberry allergic persons should not be excluded from these healthy effects, the identification of hypoallergenic strawberry varieties is an important purpose and selective breeding in this field should be increased besides the known breeding targets like fruit sensorial and nutritional traits (Mazzoni et al., 2016).

The aim of this study was to identify strawberry varieties with a low content of Fra a 1 proteins and to verify the reduced allergenic potential of these varieties by a basophil activation test (BAT). BAT is a highly specific and sensitive *in vitro* assay to detect traces of functionally active food allergens. In the BAT basophils from birch pollen allergic patients

were used to select hypoallergenic strawberry genotypes which will be beneficial for future breeding efforts. The flow cytometric BAT was applied in this study as cellular trial for *in vitro* diagnosis of IgE-mediated reactions (Eberlein et al., 2010). The BAT using strawberry extract and recombinant proteins is considered as valuable tool for the determination of sensitization to food and is routinely used for allergy clinical diagnostics (Sabato et al., 2011).

2. Materials and methods

2.1. Materials

2.1.1. Plants

The strawberry genotypes analysed by the Western blot screen are listed in Supporting Information Table S1 and were grown under the same and controlled environmental conditions in the greenhouse (Dürnast Freising, Germany). The genotypes under study for BAT were *F. vesca* red-fruited wild type (WT; undescribed accession; greenhouse Freising, Germany), *F. vesca* 'Hawaii 4', *F. vesca* cv. Yellow Wonder, *F. vesca* '185', *F. nilgerrensis* 'Dickore 1', *F. nilgerrensis* 'Dickore 2', *F. nilgerrensis* 'Mt. Omei 1', *F. nilgerrensis* 'Mt. Omei 2', *F. nilgerrensis* 'Yunnan 3', *F. nilgerrensis* 'St. 94/04-1', *F. nilgerrensis* 'St. 98/2-1', *F. nilgerrensis* 'Mt. Leigong 2', *F. × ananassa* 'P-100693', *F. × ananassa* cv. Elsanta, and *F. × ananassa* cv. Mara des Bois (Supporting information Table S1).

2.1.2. Heterologous expression and purification of *Fra a 1* proteins

Heterologous expression and purification was performed as described (Franz-Oberdorf et al., 2016; Ring et al., 2013).

2.1.3. *Fra a 1.02*-specific rabbit polyclonal antibody

The rabbit polyclonal antibody *Fra a 1.02* was raised against purified recombinant *Fra a 1.02* protein by immunizing two rabbits using an 88 d protocol. The antibody production was done by Coring System Diagnostix GmbH (Gernsheim, Germany) in collaboration with Covalab (Villeurbanne, France).

2.2. Methods

2.2.1. Screening for hypoallergenic strawberry genotypes

Strawberry fruits were harvested and stored at -20°C until protein extraction. Proteins were extracted from ripe strawberry fruits of different white- and red-fruited *Fragaria* genotypes using the phenol-extraction procedure in the presence of protease inhibitors as described (Karlsson et al., 2004). The protein concentration was measured as reported (Bradford, 1976). The proteins were separated by 12% separating SDS-PAGE under reducing conditions followed by silver staining (Blum, Beier, & Gross, 1987). Silver staining was performed for protein extracts of 42 genotypes (Supporting information Fig. S2). *Fra a 1* homologous proteins were visualized by Western blotting using a polyclonal antibody raised against *Fra a 1.02* and quantified relative to the content of *Fra a 1* proteins of a *F. vesca* red-fruited wild type in protein extracts of 51 strawberry genotypes (Franz-Oberdorf et al., 2016; Supporting information Table S1).

2.2.2. Liquid chromatography tandem MS (LC-MS/MS) analysis

Mass spectrometry was performed on an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Germany) connected to a nanoLC Ultra 1D liquid chromatography system (Eksigent, CA) using an in-house packed precolumn (20 mm $\times$ 75 μm ReproSil-Pur C18, Dr. Maisch, Germany) and analytical column (400 mm $\times$ 50 μm ReproSil-Pur C18, Dr. Maisch, Germany). After 10 min of loading and washing, peptides were transferred to the analytical column and separated using a 45 min gradient from 2% to 35% of buffer B (0.1% formic acid in acetonitrile) at 300 nL/min flow rate. The mass spectrometer was equipped with a nano-electrospray ion source (Proxeon Biosystems, DK) and the electrospray voltage was applied via a liquid junction. The mass

spectrometer was operated in data-dependent mode and all measurements were performed in positive ion mode. Intact peptide mass spectra were acquired at a resolution of 60,000 (at m/z 400) and an automatic gain control (AGC) target value of 10^6 , followed by fragmentation of the most intense ions by collision-induced dissociation (CID; LTQ Orbitrap XL). Full scans were acquired in profile mode, whereas all tandem mass spectra were acquired in centroid mode. CID was performed for up to 8 MS/MS per full scan with 35% normalized collision energy (NCE), and an AGC target value of 5000. Singly charged ions and ions without assigned charge state were excluded from fragmentation, and fragmented precursor ions were dynamically excluded (10 s). Internal calibration was performed using the poly-siloxane ion signal at m/z 445.1200 present in ambient laboratory air.

2.2.3. Peptide and protein quantification and identification

Peak lists were generated from raw MS data files using Mascot Distiller v2.4.3.1 (Matrix Science, UK) and were searched against the genome sequence of the diploid strawberry *F. vesca* 'Hawaii 4' (Shulaev et al., 2011) containing also a set of cDNA sequencing results of *Fra a 1.01E-1.08* cloned from *F. × ananassa* cv. Elsanta. The search enables contaminants and the reversed versions of all sequences with the following parameters: carbamidomethylation of cysteine residues as fixed modification and acetyl (protein N-term) and methionine oxidation as variable modifications. Trypsin was specified as the proteolytic enzyme and up to two missed cleavages were allowed. The mass tolerance of the precursor ion was set to 20 ppm and for fragmentations to 0.5 Da. Data interpretation was performed using Scaffold 4, v.4.2.1 (Searle, 2010). All proteins were filtered under a false discovery rate of 0.0%.

2.2.4. Atopic and non-atopic groups

The selection of atopic and non-atopic individuals and their clinical and biological characterisation was performed as described (Franz-Oberdorf et al., 2016). All patients were without medication against birch pollen allergy for at least 14 days before blood sampling. The basophil activation tests were performed outside the birch pollen season, which lasted in Munich from April 4th to May 27th 2013. Data were collected in February, March and June 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 (Franz-Oberdorf et al., 2016).

2.2.5. Basophil activation test by flow cytometry

The recombinant protein Bet v 1a (*Betula verrucosa 1a*) was obtained from Biomay (Vienna, Austria) (Schober et al., 2006). A commercially available test system (Flow CAST® 2011, Bühlmann Laboratories AG, Schönenbuch, Switzerland) was used for the quantitative determination of basophil activation by Bet v 1a and 15 protein samples from red- and naturally occurring white-fruited strawberry varieties by flow cytometry, as previously described (Eberlein et al., 2010; Franz-Oberdorf et al., 2016). Whole blood samples were collected into 10 ml K-EDTA vein puncture tubes and put on a vortex mixer at 22°C . The tests were carried out within 24 h of blood sampling. The recombinant protein Bet v 1a and the strawberry fruit protein extracts were dissolved in ultrapure water to a concentration of 100 $\mu\text{g}/\text{ml}$ and used at five different concentrations (1000 ng/ml, 500 ng/ml, 250 ng/ml, 125 ng/ml, and 62.5 ng/ml). A negative control without allergen, and both positive controls (anti-Fc ϵ RI and fMLP) included in the Flow CAST® kit were treated like the samples with allergen. The basophil activation test was performed following the manual instructions of Flow CAST®. Flow cytometric analysis was performed within 2 h 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 (Eberlein, Krischan, Darsow, Ollert, & Ring, 2012; Franz-Oberdorf et al., 2016; Schober et al., 2006).

2.2.6. Statistical analysis

The boxplot was created by using the software R (The R Foundation for Statistical Computing, R version 2.13.0). Statistical significance levels between the different allergens, subject groups and controls were calculated using one-way analysis of variance (ANOVA). A p value of ≤ 0.05 was considered as statistically significant. Data points were expressed as the arithmetic means $\pm$ SD. ImageJ (<https://imagej.nih.gov/ij/>) was used to determine the relative intensities of allergen bands detected by Western blotting normalized against the control *F. vesca* red-fruited WT (was set 100%).

2.2.7. Immuno-tissue-prints (ITP)

ITP-assays were performed as described (Marzban et al., 2005). Since strawberry fruits released a lot of liquid after cutting a filter paper was used to remove the liquid before printing the cut surface on the pre-treated polyvinylidene fluoride (PVDF) membrane. Fra a 1 homologous proteins on the PVDF membrane were visualized using the polyclonal antibody raised against Fra a 1.02 in rabbit and the secondary anti-rabbit antibody conjugated with AP by Western blotting.

3. Results

3.1. Identification of putative hypoallergenic strawberry varieties

Because a naturally occurring white-fruited strawberry genotype was tolerated by allergic individuals and lacked the allergen Fra a 1 and major enzymes of the anthocyanin biosynthesis pathway (Hjernø et al., 2006) we started a screening of 51 white- and red-fruited strawberry species to discover additional hypoallergenic strawberry varieties (Supporting information Table S1). Protein extracts of *F. vesca* red-fruited wild type, *F. × ananassa* cultivars Elsanta and Mara des Bois served as controls. The protocol used for protein extraction is appropriate to isolate in particular small proteins like Fra a 1 isoforms (Karlsson et al., 2004). The protein extracts (5 µg each) were separated by SDS-PAGE (continuous 12% separating gel) and analysed by silver staining and Western blot analysis using a polyclonal antibody directed to Fra a 1.02. The Western blot screen was performed twice with biological replicates. Three clear bands were detected by Western blot analysis in nearly all strawberry protein extracts at approx. 15, 18, and 20 kDa (Supporting information Fig. S1). The proteins contained in the bands were partially sequenced by mass spectrometry. The detected amino acid sequences were compared with the translated *F. vesca* genome sequence (Shulaev et al., 2011) and *F. × ananassa* cv. Elsanta protein sequences of Fra a 1.01E-1.08 (Franz-Oberdorf et al., 2016; Searle, 2010). Both protein spots at 18 and 20 kDa contained the peptide AFVLDADNLIPK and additional unique peptides of Fra a 1 proteins yielding 68% sequence coverage. This result is in good accordance with both, the *in silico* calculated molecular mass of the Fra a 1 proteins (17.3–21.7 kDa) (Hyun & Kim, 2011) and the previous analysis of strawberry allergens (Karlsson et al., 2004). The protein spot at 15 kDa exhibited unique peptides for calmodulin-2/3/5 (gene10223) (Shulaev et al., 2011).

Out of 51 different strawberry fruit protein extracts, eleven white-fruited genotypes showed considerable lower levels of both Fra a 1 proteins at 18 and 20 kDa when compared with the extract from *F. vesca* red-fruited wild type control (Supporting information Table S1). However, the white-fruited *F. × ananassa* 'P-100693' and the red-fruited *F. × ananassa* cultivars Elsanta and Mara des Bois produced higher amounts of the Fra a 1 proteins than the *F. vesca* red-fruited wild type control. Twelve white-fruited strawberry genotypes, eleven with lower level of Fra a 1 proteins and *F. × ananassa* 'P-100693' with a higher level of Fra a 1 proteins, were included into the BAT trial in addition to the positive controls *F. vesca* red-fruited wild type, *F. × ananassa* cv. Elsanta and *F. × ananassa* cv. Mara des Bois. The 15 strawberry genotypes used in the BAT trial were grown under the same and controlled environmental conditions in the greenhouse (Dürnast

Freising, Germany) (Supporting information Table S1).

3.2. Characterisation of patients

The selection of atopic and non-atopic individuals as well as their clinical and biological characterisation was the same as described in (Franz-Oberdorf et al., 2016). The eight atopic patients with *Rhinoconjunctivitis allergica* against birch pollen showed partly positive clinical reactions (OAS) against apple, tomato, and other related allergen harbouring fruits and vegetables. Two patients had a clear-cut history of strawberry allergy whereas two others of the eight atopic individuals 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 skin prick test (SPT) using the commercially available strawberry extract. The prick to prick test was negative in all patients with *F. vesca* red-fruited wild type, positive in four cases with *F. × ananassa* cv. Mara des Bois and positive in six cases with *F. × ananassa* 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 or strawberry and showed a negative SPT to birch pollen (data not shown) and the different strawberry extracts. The data were collected in 2013 but outside the birch pollen season.

3.3. Validation of the basophil activation test (BAT)

Basophils of patients included in the present study were identified by flow cytometry. They were separated from the other blood cells using the selection marker CCR3 and are shown in the left upper square in Fig. 1a–e. Activated basophils tagged with anti-CD63 FITC antibody were counted in a population of 500 gated basophils and are located in the upper right quadrant (Q2) of the dot plots (Fig. 1f–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 recombinant Bet v 1a, proteins of 15 naturally occurring red- and white-fruited strawberry varieties, two positive (anti-FcεRI, fMLP) and a negative control of the kit Flow CAST® (Table 1).

The allergenic potential of the extracts of ten varieties displayed a clear dose-response curve with the highest basophil activation upon stimulation with the highest concentration of 1000 ng/ml (Supporting information Fig. S2). The remaining five sample types (*F. vesca* '185', *F. nilgerrensis* 'Mt. Omei 2', *F. nilgerrensis* 'St. 98/2-1', *F. nilgerrensis* 'Mt. Leigong 2', *F. × ananassa* cv. Elsanta) exhibited a different curve with the highest basophil activation at a concentration of 250 ng/ml or 500 ng/ml. The allergen Bet v 1a served as positive control with the highest activation of basophils at a concentration of 500 ng/ml. Basophils of the control group (non-atopic individuals) were not activated by stimulation neither with Bet v 1a or the extracts of 15 different strawberry varieties. However, activation of basophils isolated from atopic donors by strawberry fruit extracts was clearly observed at 1000 ng/ml (Supporting information Fig. S2; Table 1).

The reliability of the method was confirmed by the analysis of control samples. The mean percentage of CD63 + basophils after incubation with both positive controls of the experimental system anti-FcεRI (highly specific monoclonal antibody binding to the high affinity IgE binding receptor) and fMLP (unspecific cell activator) in eight patients with a history of RA against birch pollen was 79.56% ($\pm$ 0.19%) and 40.77% ($\pm$ 0.30%), respectively compared with an average value of 82.82% ($\pm$ 0.13%) and 29.17% ($\pm$ 0.30%) in the control group (Table 1). Similarly, the results of the negative control (blank sample) in the group of atopic patients differed not significantly from the results in the control group. The reliability results were similar to the data described (Franz-Oberdorf et al., 2016).

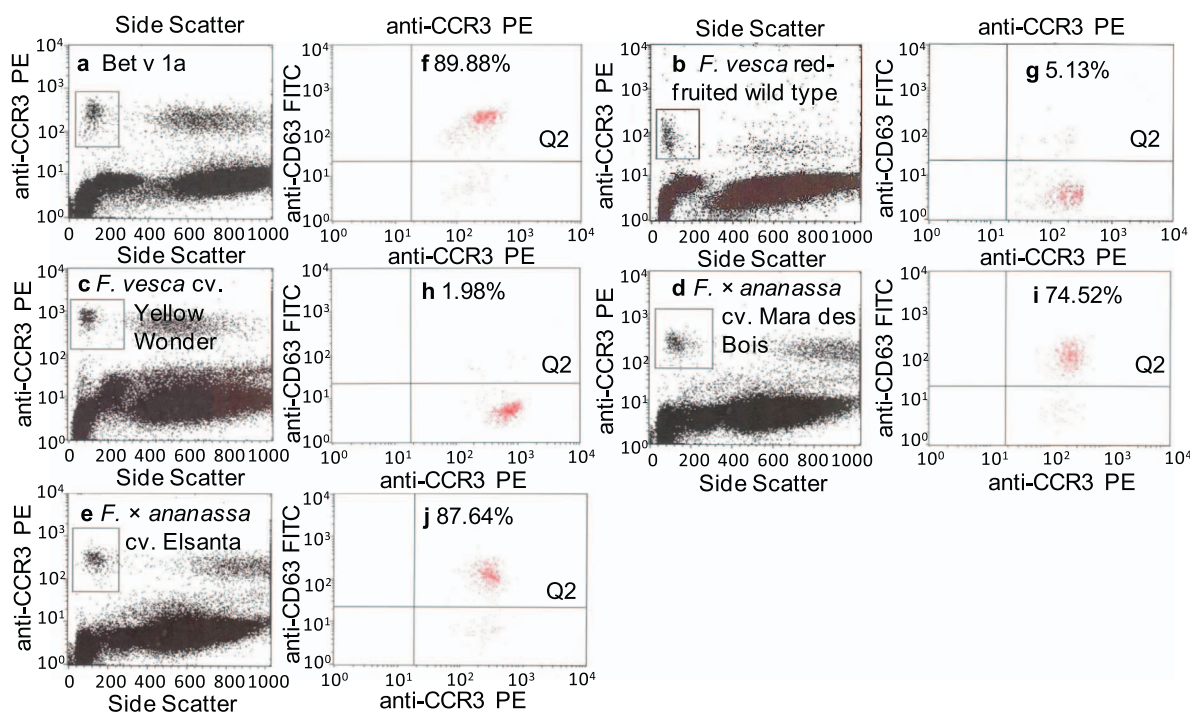


Fig. 1. Flow cytometric analysis. Dot plot examples of basophil gating using CCR3 selection marker in basophils from a birch pollen allergic individual after incubation with the positive control Bet v 1a (a), a phenolic extract of *F. vesca* red-fruited wild type (b), *F. vesca* cv. Yellow Wonder (c), *F. x ananassa* cv. Mara des Bois (d) and *F. x ananassa* cv. Elsanta (e) at a concentration of 1000 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.

3.4. BAT of protein extracts from different strawberry varieties

The percentages of basophils activated by protein extracts of different strawberry varieties at 1000 ng/ml concentration, including positive and negative controls were visualized as box plots (Fig. 2a). The positive controls Bet v 1a, anti-FcεRI, and fMLP and a negative control included in the Flow CAST® kit confirmed the system stability. The basophil stimulation with Bet v 1a, the selection criterion for allergic individuals, led to CD63 + saturation with a median at about 56% and showed the high biological variability within the atopic patient group (Fig. 2a). Consistent with the observation that the allergics showed strong symptoms against the protein extracts from *F. x ananassa* cultivars Elsanta and Mara des Bois in SPT (Franz-Oberdorf et al.,

2016), proteins from octoploid *F. x ananassa* cultivars, including variety ‘P-100693’ activated a high percentage of basophils (62–74%) with little biological variability, except ‘P-100693’ (Fig. 2a).

The white-fruited *F. vesca* ‘Hawaii 4’ and cv. Yellow Wonder, *F. nilgerrensis* ‘Dickore 1’, ‘Dickore 2’, ‘Mt. Omei 1’, ‘Yunnan 3’ and the red-fruited *F. vesca* wild type showed high biological variability across the atopic group. However, *F. vesca* ‘185’, *F. nilgerrensis* ‘Mt Omei 2’, ‘St. 94/04-1’, ‘St. 98/2-1’, and ‘Leigong 2’ displayed little biological variation and activated $> 85\%$ of the basophils. Notably, median percentage of activated basophils was lowest for *F. vesca* cv. Yellow Wonder (36%) in the entire data set. This observation led to the conclusion that *F. vesca* cv. Yellow Wonder shows a low allergenic potential for patients suffering from an allergy against Bet v 1-related proteins. In contrast, *F.*

Table 1

CD63 + basophil activation of atopic (n = 8) and non-atopic (n = 2) donors by recombinant Bet v 1a and protein extracts from different natural occurring white- and red-fruited strawberry cultivars (c = 1000 ng/ml), two positive controls and a blank value. (* white fruited strawberry cultivars, *Fragaria* varieties without star produce fruits).

Stimulus	CD63 + mean [%] atopic group	SD [%] atopic group	CD63 + mean [%] nonatopic group	SD [%] nonatopic group
Bet v 1a	55.39	0.21	0.20	0.002
<i>F. vesca</i> red-fruited wild type	44.44	0.40	0.52	0.004
<i>F. vesca</i> ‘Hawaii 4’*	54.45	0.35	1.58	0.01
<i>F. vesca</i> cv. Yellow Wonder*	36.35	0.34	0.26	0.0005
<i>F. vesca</i> ‘185’*	75.93	0.27	0.84	0.003
<i>F. nilgerrensis</i> ‘Dickore 1’*	56.70	0.37	0.33	0.0007
<i>F. nilgerrensis</i> ‘Dickore 2’*	54.85	0.40	1.07	0.002
<i>F. nilgerrensis</i> ‘Mt. Omei 1’*	46.01	0.34	0.80	0.003
<i>F. nilgerrensis</i> ‘Mt. Omei 2’*	81.98	0.15	0.32	0.004
<i>F. nilgerrensis</i> ‘Yunnan 3’*	59.36	0.41	0.74	0.007
<i>F. nilgerrensis</i> ‘St.94/04-1’*	80.82	0.24	1.84	0.01
<i>F. nilgerrensis</i> ‘St.98/2-1’*	79.03	0.27	0.51	0.003
<i>F. nilgerrensis</i> ‘Leigong 2’*	84.16	0.17	3.07	0.02
<i>F. x ananassa</i> ‘P-100693’*	53.42	0.31	0.40	0.003
<i>F. x ananassa</i> cv. Elsanta	67.12	0.20	0.50	0.004
<i>F. x ananassa</i> cv. Mara de Bois	75.84	0.10	0.48	0.007
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

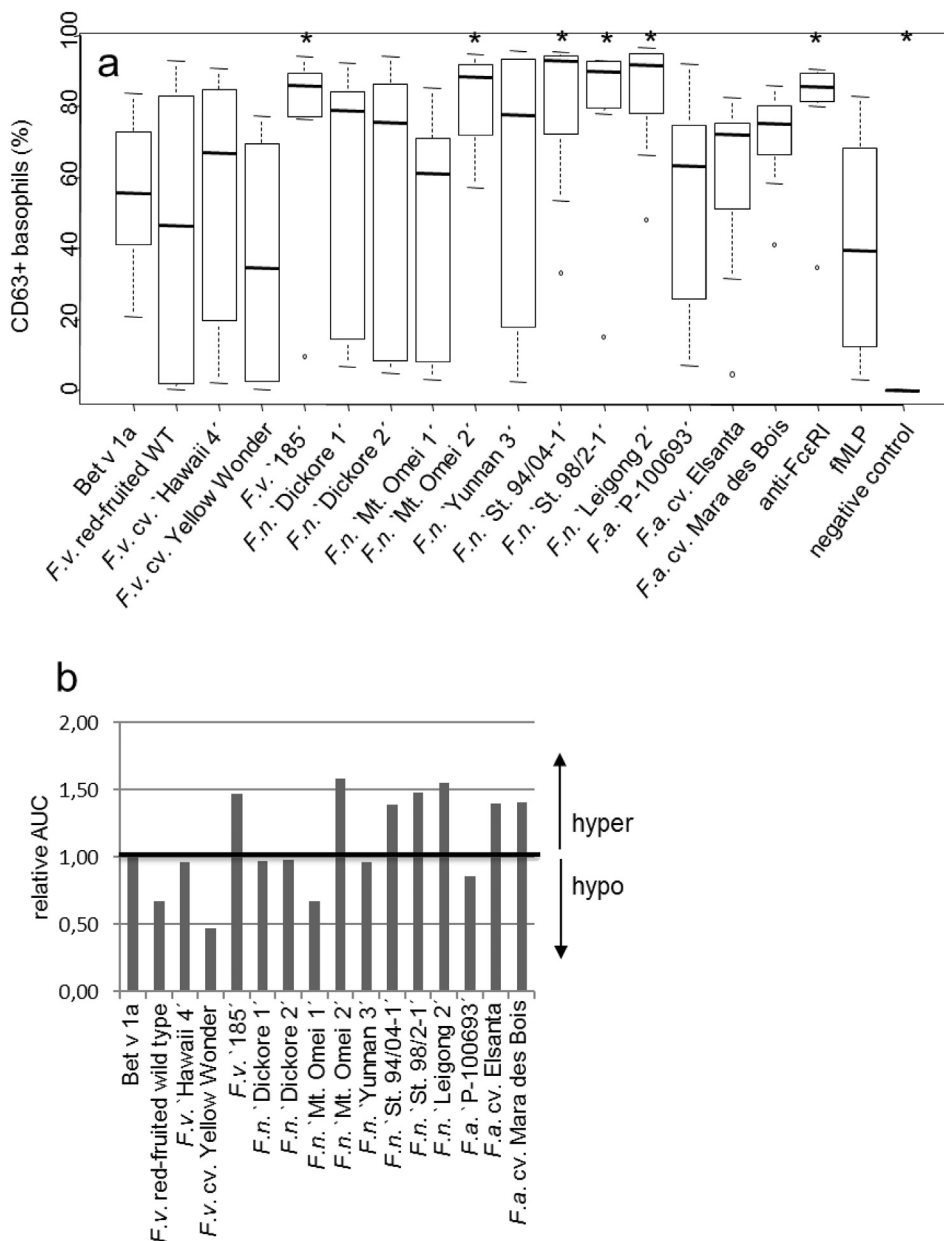


Fig. 2. Basophil activation in the BAT with extracts of natural occurring white- and red-fruited strawberry genotypes. (a) Basophil activation in the BAT in birch pollen allergic ($n = 8$) after incubation with Bet v 1a and phenolic extracts of natural occurring white- and red-fruited varieties of *Fragaria vesca* (F. v.), *Fragaria nilgerrensis* (F. n.) and *Fragaria × ananassa* (F. a.), including two positive controls (anti-FcεRI and fMLP) and the blank value negative control at a concentration of 1000 ng/mL. (*) p-value < 0.05 referred to Bet v 1a. (b) Relative area under the curve (AUC) of the activated CD63 + basophils results of all analysed samples. AUC of the samples are normalized against Bet v 1a.

vesca '185', *F. nilgerrensis* 'Mt. Omei 2', 'St. 94/04-1', 'St. 98/2-1', and 'Leigong 2' were considered as allergenic strawberry lines. They revealed a statistically significant higher allergic potential compared with the data for Bet v 1a (Fig. 2a, Table 1).

The differentiation of the strawberry varieties according to their ability to activate basophils is also supported by the relative AUC values that take into account the concentration dependence of the BAT (Fig. 2b). *F. vesca* cv. Yellow Wonder exhibited the lowest AUC value in the experiment and can be regarded as hypoallergenic strawberry variety, whereas *F. vesca* '185', *F. nilgerrensis* 'Mt. Omei 2', 'St. 94/04-1', 'St. 98/2-1', 'Leigong 2', and *F. × ananassa* cv. Elsanta and cv. Mara des Bois showed the highest AUC values and are strawberry varieties that bear a higher risk to trigger allergic reactions.

3.5. Immuno tissue printing

An ITP experiment was performed with ripe, turning (intermediate stage) and green fruits from *F. vesca* cv. Yellow Wonder, *F. vesca* red-fruited wild type, *F. × ananassa* cv. Elsanta and cv. Mara des Bois to

localize Bet v 1-related allergens in strawberry fruit. Fra a 1 proteins were visualized by a polyclonal antibody raised against Fra a 1.02. Fra a 1 proteins were almost evenly distributed in fruits of the three maturity stages and the four varieties (Supporting information Fig. S3). More intense signals were obtained when a greater pressure was applied while printing the tissue (*F. vesca* red-fruited WT, Supporting Information Fig. S3b). Faint signals were produced when Fra a 1 proteins of *F. vesca* cv. Yellow Wonder were stained. *F. × ananassa* cv. Elsanta and cv. Mara des Bois yielded strong signals.

4. Discussion

Birch pollen allergics often suffer from adverse reactions to *Rosaceae* fruits in Central and Northern Europe because of the presence of cross-reactive allergens (Hauser, Roulias, Ferreira, & Egger, 2010; Vieths, Scheurer, & Ballmer-Weber, 2002). Proteins, structurally related to the major birch pollen allergen Bet v 1 and other members of the PR-10 family have been isolated e.g. from apple (Mal d 1) and strawberry fruit (Fra a 1) (Laimer & Maghuly, 2010; Rodriguez et al., 2000). Thus, in a

former study, four and six out of eight atopic donors showed a positive SPT reaction against protein extracts from fruits of the cultivated genotypes *F. × ananassa* cultivars Mara des Bois and Elsanta, respectively. When an extract from the wild progenitor *F. vesca* was used wheal and flare reactions were less prominent (Franz-Oberdorf et al., 2016). The same panel of volunteers was recruited for the present study.

Bet v 1 homologous proteins are ubiquitous in the plant kingdom and are described as panallergens (Karlsson et al., 2004; Marzban et al., 2005). The reactions seen in birch pollen allergic patients against food are primarily explained by IgE antibodies to Bet v 1, induced by birch pollen. These antibodies can cross-react with Bet v 1 homologous proteins in different plant derived foods. A high percentage of birch pollen allergics are sensitized to Bet v 1 in the birch tree-rich areas of Northern and Central Europe. Most of them have Bet v 1-IgE antibodies as the only specificity. Bet v 1 homologues are primarily found in the pulp of the fruit, almost evenly distributed (Erdmann et al., 2005), as was also demonstrated for strawberry fruit of different varieties (Supporting information Fig. S3). In areas where birch trees are rare, such as Southern Europe adverse reactions to birch pollen often reflect sensitization to Bet v 1-related proteins from other trees closely related to birch (e.g. alder, hazel, and hornbeam) or sensitization to other pollen allergen families such as profilins (Bet v 2 homologues; Fra a 4) in grass and weed (Vieths et al., 2002).

4.1. Allergenic potential of strawberry genotypes

To identify low-allergenic strawberry cultivars a Western blot screen of 51 genotypes was performed with an antibody raised against Fra a 1.02. Three clear bands at about 15, 18, and 20 kDa were visualized by the antibody (Supporting information Fig. S1). The appearance of the Fra a 1 proteins in two distinct separated bands can be explained by post-translational modifications, splice variants or allelic versions of the strawberry proteins. The octoploid strawberry *F. × ananassa* cultivars express up to 8 alleles of a *Fra a 1* gene (Hjerno et al., 2006) whose encoded proteins could not be clearly separated (Supporting information Fig. S1). The third band contained a different protein (calmodulin-2/3/5, gene 10,223) (Shulaev et al., 2011). It seems that calmodulin-2/3/5 and Fra a 1 possess a similar partial protein sequence or tertiary structure that may serve as epitopes for the polyclonal antibody raised against Fra a 1.02.

Eleven white-fruited strawberry genotypes were selected for further analyses by BAT due to the low abundance of the protein bands at 18 and/or 20 kDa in the Western blot screen (Supporting information Table S1). Besides, *F. vesca* red-fruited wild type and *F. × ananassa* cultivars P-100693 (white-fruited), Elsanta and Mara des Bois served as controls. Despite low signal intensities of the Fra a 1 proteins in the Western blot screen the BAT demonstrated a considerable high allergic potential for protein extracts from white-fruited *F. nilgerrensis* genotypes, *F. vesca* varieties ('Hawaii 4' and '185') and *F. × ananassa* 'P100693' (Fig. 2). Thus, it can be assumed that white-fruited strawberry varieties are not generally tolerated by allergic individuals although one hypoallergenic line has been reported (Hjerno et al., 2006).

The results show that an allergic reaction of an atopic individual is highly complex and eliciting allergens often involve more than one protein family. In addition to Fra a 1, protein families such as Fra a 3 from the plant nsLTP1 (non-specific lipid transfer protein) group which is a subgroup of the PR-14 protein family and Fra a 4 (profilins) play also an important role in allergic reactions induced by strawberry fruit extracts (Andersen, Hall, & Dragsted, 2011; Laimer & Maghuly, 2010). The panallergen profilin, the eurallergen nsLTP and PR-10 proteins lead to clinically relevant IgE cross-reactivity between unrelated allergen sources which occur in pollen, plant food and products (Hauser et al., 2010). In addition, cross-reactivity between the protein families Bet v 1 (PR-10) and Bet v 2 (profilin) was observed by a cross-inhibition caused by a profilin-specific antibody (Ebner et al., 1995). Thus, the unexpected high allergenic potential of fruit of *F. nilgerrensis*, some *F. vesca*

and *F. × ananassa* cultivars may also be attributed to allergy-causing Fra a 3 and Fra a 4 proteins. On the other hand it should be noted that extensive IgE cross-reactivity among PR-10 proteins, nsLTPs, profilins and further unrelated proteins could also be caused by cross-reactive carbohydrate determinants (CCD) of glycoproteins which are widely distributed across evolutionary lineages (Bishop & Gagneux, 2007; Hauser et al., 2010; Macher & Galili, 2008). These factors might contribute to the high biological variability of CD63 + basophil activation when stimulated by strawberry fruit extracts (Fig. 2). Overall, only *F. vesca* cv. Yellow Wonder showed a minor allergenic potential by Western blot and BAT, respectively (Table 1, Figs. 1–2).

4.2. Transcript analysis of Fra a 1 genes

Recently, the gene transcript levels of the strawberry fruit allergens *Fra a 1.01E*, *1.02*, and *1.03* were determined in fruits of *F. vesca* red-fruited wild type, *F. vesca* cv. Yellow Wonder, *F. × ananassa* cultivars Elsanta and Mara des Bois (Franz-Oberdorf et al., 2016). The highest expression level of the gene *Fra a 1.02*, encoding the most potent isoform of the Bet v 1-like allergen was found in fruits of *F. × ananassa* cultivars Elsanta and Mara des Bois and was 110- and 220-fold, respectively higher than in the *F. vesca* red-fruited wild type and *F. vesca* cv. Yellow Wonder genotype, respectively (Franz-Oberdorf et al., 2016). Thus, it appears that activation of basophils by protein extracts from different strawberry genotypes correlates with the expression level of the *Fra a 1.02* gene in the fruits and confirms the allergenic potency of the isoforms Fra a 1.02 and thus the minor allergenic potential of the genotype *F. vesca* cv. Yellow Wonder.

4.3. Mutations underlying the fruit colour

In *Fragaria*, red-, yellow- and white-fruited accessions exist. High levels of anthocyanins are present in the red fruit but not in the yellow and white fruit (Urrutia, Schwab, Hoffmann, & Monfort, 2016). Co-segregation between an anthocyanin biosynthesis gene *Flavanone 3-Hydroxylase (F3H)* and the locus C (yellow fruit) was observed and led to the hypothesis that *F3H* is a candidate gene for locus c (Deng & Davis, 2001). In *F. × ananassa* and *F. vesca* the transcription factor *FaMYB10* was shown to play critical roles in regulating the accumulation of red pigment in the receptacle (Hawkins et al., 2016) as RNAi down-regulation of *FaMYB10* resulted in significant reduction of anthocyanin in the receptacle fruit (Medina-Puche et al., 2014). Similarly, down-regulation of abscisic acid biosynthesis genes produced fruit with impaired anthocyanin accumulation (Jia et al., 2011). In *F. chiloensis* subsp. *chiloensis* anthocyanidin glucosyltransferase seems to be mutated (Griesser et al., 2008). Transcriptome sequencing of *F. vesca* cv. Yellow Wonder showed that transcript levels of several anthocyanin biosynthesis genes and several MYB genes are reduced in the *F. vesca* cv. Yellow Wonder fruit (Haertl et al., 2017; Zhang et al., 2015). Besides, fruit of a colourless (white-fruited) unknown strawberry variety were tolerated by individuals affected by allergy because the fruit were nearly free from the strawberry allergen Fra a 1 (Hjerno et al., 2006). Proteome analysis revealed that the levels of several enzymes of the anthocyanin biosynthesis pathway (Griesser et al., 2008; Hjerno et al., 2006; Muñoz et al., 2010) were also reduced and RNAi down-regulation of *Fra a 1* genes resulted in a considerable reduction of anthocyanin pigment (Muñoz et al., 2010). These examples demonstrate that mutations underlying the fruit colour are manifold and lead to the conclusion that white-fruited strawberry fruit do not consistently lack Fra a 1 allergens. The cultivation of hypoallergenic cultivars should become the most favourable purpose in the field of strawberry selective breeding besides other breeding targets like fruit sensorial and nutritional traits (Mazzoni et al., 2016).

Our results reveal that fruit of *F. vesca* cv. Yellow Wonder show low allergenic potential and might be accepted by patients with mild allergy. The data are valuable for the selective breeding of hypoallergenic

strawberry cultivars.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.foodres.2017.07.076>.

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Supporting Information

White-fruited strawberry genotypes are not per se hypoallergenic

Katrin Franz-Oberdorf¹, Bernadette Eberlein², Kathrin Edelmann¹, Philip Bleicher¹, Dominic Helm³, Klaus Olbricht⁴, Ulf Darsow², Johannes Ring², Wilfried Schwab^{1*},

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

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

³Chair of Proteomics and Bioanalytics, Technische Universität München, Freising, Germany

⁴The Strawberry Breeding Company Hansabred GmbH & Co. KG, Radeburger Landstr. 12, 01108 Dresden, Germany

Table S1. Red- and white-fruited strawberry genotypes used for the Western blot screen. Relative intensities of the 18 and 20 kDa bands of the Western blot screen were determined by ImageJ (bands of *Fragaria vesca* red-fruited wild type was set 100%). Genotypes shown in bold were selected for basophil activation test (BAT).

No.	species	propagation (study)	Propagation (supplier)	supplier
1	<i>F. vesca</i> red-fruited wild type (red)	reference (set to 100%)	greenhouse	Dürnast Freising
2	<i>F. vesca</i> 'Hawaii 4'	13/45	greenhouse	Dürnast Freising
3	<i>F. vesca</i> cv. Yellow Wonder	83/23	field	Hansabred Dresden
4	<i>F. vesca</i> '185'	63/58	greenhouse	University of Malaga
5	<i>F. vesca</i> cv. Red Wonder (red)	91/111	field	University of Malaga
6	<i>F. vesca</i> 'Blanca'	87/111	greenhouse	University of Talca, Chile
7	<i>F. vesca</i> 'Reina de los Vallas Roja' (red)	73/93	greenhouse	University of Talca, Chile
8	<i>F. vesca</i> 'Moritzburg'	57/46	greenhouse	Julius Kühn Institut Dresden
9	<i>F. vesca</i> 'forma alba St. 08/101'	57/32	greenhouse	Julius Kühn Institut Dresden
10	<i>F. vesca</i> 'forma alba S. Queen Ferry'	43/37	greenhouse	Julius Kühn Institut Dresden
11	<i>F. vesca</i> '156'	181/296	greenhouse	University of Malaga
12	<i>F. vesca</i> '285'	101/119	greenhouse	University of Malaga
13	<i>F. vesca</i> '596'	225/158	greenhouse	University of Malaga
14	<i>F. vesca</i> '175'	118/123	greenhouse	University of Malaga
15	<i>F. vesca</i> '412'	68/85	greenhouse	University of Malaga
16	<i>F. vesca</i> '425'	79/117	greenhouse	University of Malaga
17	<i>F. nilgerrensis</i> 'Dickore 1'	28/17	greenhouse	Hansabred Dresden
18	<i>F. nilgerrensis</i> 'Dickore 2'	25/15	greenhouse	Hansabred Dresden
19	<i>F. nilgerrensis</i> 'Mt. Omei 1'	25/23	greenhouse	Hansabred Dresden
20	<i>F. nilgerrensis</i> 'Mt. Omei 2'	71/90	greenhouse	Hansabred Dresden
21	<i>F. nilgerrensis</i> 'Yunnan 3'	13/19	greenhouse	Hansabred Dresden
22	<i>F. nilgerrensis</i> 'Yunnan 4'	31/39	greenhouse	Hansabred Dresden
23	<i>F. nilgerrensis</i> 'St. 94/04-1'	56/46	greenhouse	Hansabred Dresden
24	<i>F. nilgerrensis</i> 'St. 98/2-1'	18/8	greenhouse	Hansabred Dresden
25	<i>F. nilgerrensis</i> 'Leigong 1'	112/126	greenhouse	Hansabred Dresden
26	<i>F. nilgerrensis</i> 'Leigong 2'	36/36	greenhouse	Hansabred Dresden
27	<i>F. nilgerrensis</i> 'Apricot Chinoise'	131/142	greenhouse	Hansabred Dresden
28	<i>F. nilgerrensis</i> '77'	73/83	greenhouse	Julius Kühn Institut Dresden
29	<i>F. nilgerrensis</i> '78'	46/60	greenhouse	Julius Kühn Institut Dresden
30	<i>F. x ananassa</i> cv. Elsanta (red)	206/207	greenhouse	Dürnast Freising
31	<i>F. xananassa</i> cv. Mara des Bois (red)	227/169	greenhouse	Dürnast Freising
32	<i>F. xananassa</i> 'P-100693'	190/157	greenhouse	Hansabred Dresden
33	<i>F. xananassa</i> 'P-100693 type 2'	183/192	field	Hansabred Dresden
34	<i>F. xananassa</i> cv. Anablanca	167/269	greenhouse	Hansabred Dresden
35	<i>F. xananassa</i> cv. White Pine	214/247	greenhouse	Hansabred Dresden
36	<i>F. xananassa</i> cv. White Dream	151/271	field	Hansabred Dresden
37	<i>F. xananassa</i> cv. White Ananas	150/210	field	Hansabred Dresden
38	<i>F. xananassa</i> 'P-90000'	131/171	field	Hansabred Dresden
39	<i>F. xananassa</i> 'P-90000 type 2'	99/137	greenhouse	Hansabred Dresden
40	<i>F. xananassa</i> 'P-100120'	110/128	greenhouse	Hansabred Dresden
41	<i>F. xananassa</i> 'P-100121'	71/79	field	Hansabred Dresden
42	<i>F. xananassa</i> 'P-100122'	170/219	field	Hansabred Dresden
43	<i>F. xananassa</i> 'P-100123'	85/115	field	Hansabred Dresden
44	<i>F. xananassa</i> 'P-100125'	70/104	field	Hansabred Dresden
45	<i>F. xananassa</i> 'P-100675'	80/106	field	Hansabred Dresden
46	<i>F. xananassa</i> 'P100677'	153/212	greenhouse	Hansabred Dresden
47	<i>F. xananassa</i> 'P100677 type 2'	133/213	field	Hansabred Dresden
48	<i>F. xananassa</i> 'P-100678'	221/363	field	Hansabred Dresden
49	<i>F. xananassa</i> 'P-100691'	107/127	field	Hansabred Dresden
50	<i>F. xananassa</i> 'P-100695'	111/156	field	Hansabred Dresden
51	<i>F. xananassa</i> 'P-100696'	159/199	field	Hansabred Dresden

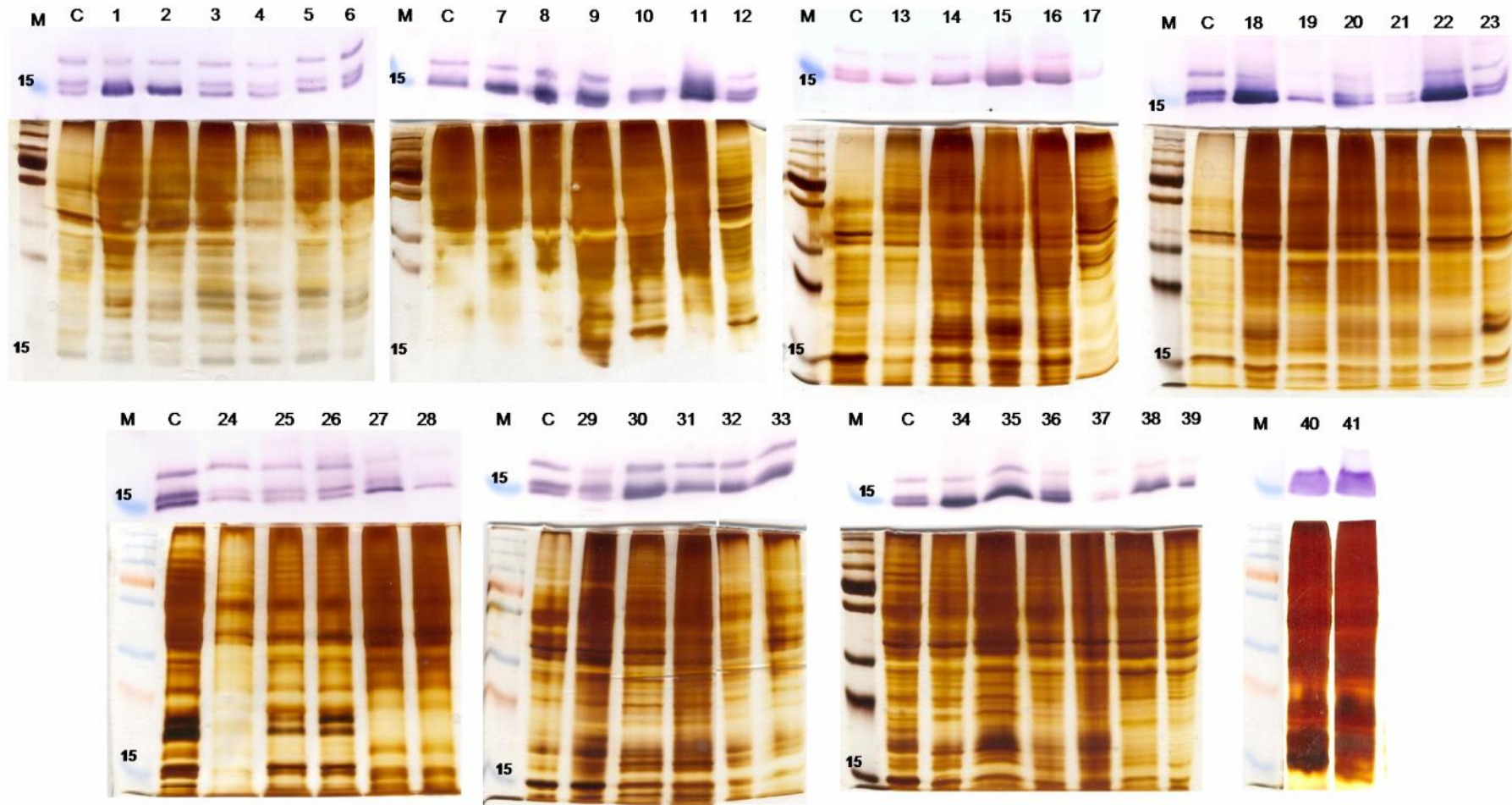


Figure S1. Silver stained SDS-PAGE and Western blot analysis of a phenolic extract of different strawberry varieties (5 µg protein each). Western blot was performed using the polyclonal Fra a 1.02 antibody. M: protein marker: PageRuler Plus Prestained Protein Ladder (Thermo Scientific); C: *F. vesca* red-fruited ET; (1) *F. x ananassa* cv. White Pine, (2) *F. x ananassa* cv. White Ananas, (3) *F. vesca* '175', (4) *F. vesca* '185', (5) *F. vesca* '412', (6) *F. vesca* '425', (7) *F. x ananassa* 'P100695', (8) *F. x ananassa* 'P100122', (9) *F. vesca* '156', (10) *F. vesca* '285', (11) *F. vesca* '596', (12) *F. x ananassa* 'P100121', (13) *F. x ananassa* 'P100123', (14) *F. x ananassa* 'P100125', (15) *F. x ananassa* 'P100693', (16) *F. x ananassa* 'P90000', (17) *F. nilgerrensis* 'Yunnan 3', (18) *F. x ananassa* cv. White Dream, (19) *F. nilgerrensis* 'Yunnan 4', (20) *F. nilgerrensis* 'Mt. Omei 2', (21) *F. nilgerrensis* 'Mt. Omei 1', (22) *F. x ananassa* cv. Anablanca, (23) *F. vesca* 'forma alba St. 08/101', (24) *F. nilgerrensis* 'Dickore 1', (25) *F. vesca* 'forma alba S. Queen Ferry', (26) *F. vesca* 'Moritzburg', (27) *F. nilgerrensis* 'St. 94/04-1', (28) *F. nilgerrensis* 'St. 98/2-1', (29) *F. vesca* cv. Yellow Wonder, (30) *F. x ananassa* 'P100677', (31) *F. x ananassa* 'P100120', (32) *F. x ananassa* 'P100675', (33) *F. x ananassa* 'P100691', (34) *F. x ananassa* 'P100696', (35) *F. x ananassa* 'P100678', (36) *F. nilgerrensis* 'Apricot Chinoise', (37) *F. nilgerrensis* 'Dickore 2', (38) *F. nilgerrensis* 'Leigong 1', (39) *F. nilgerrensis* 'Leigong 2', (40) *F. x ananassa* cv. Mara des Bois, (41) *F. x ananassa* cv. Elsanta.

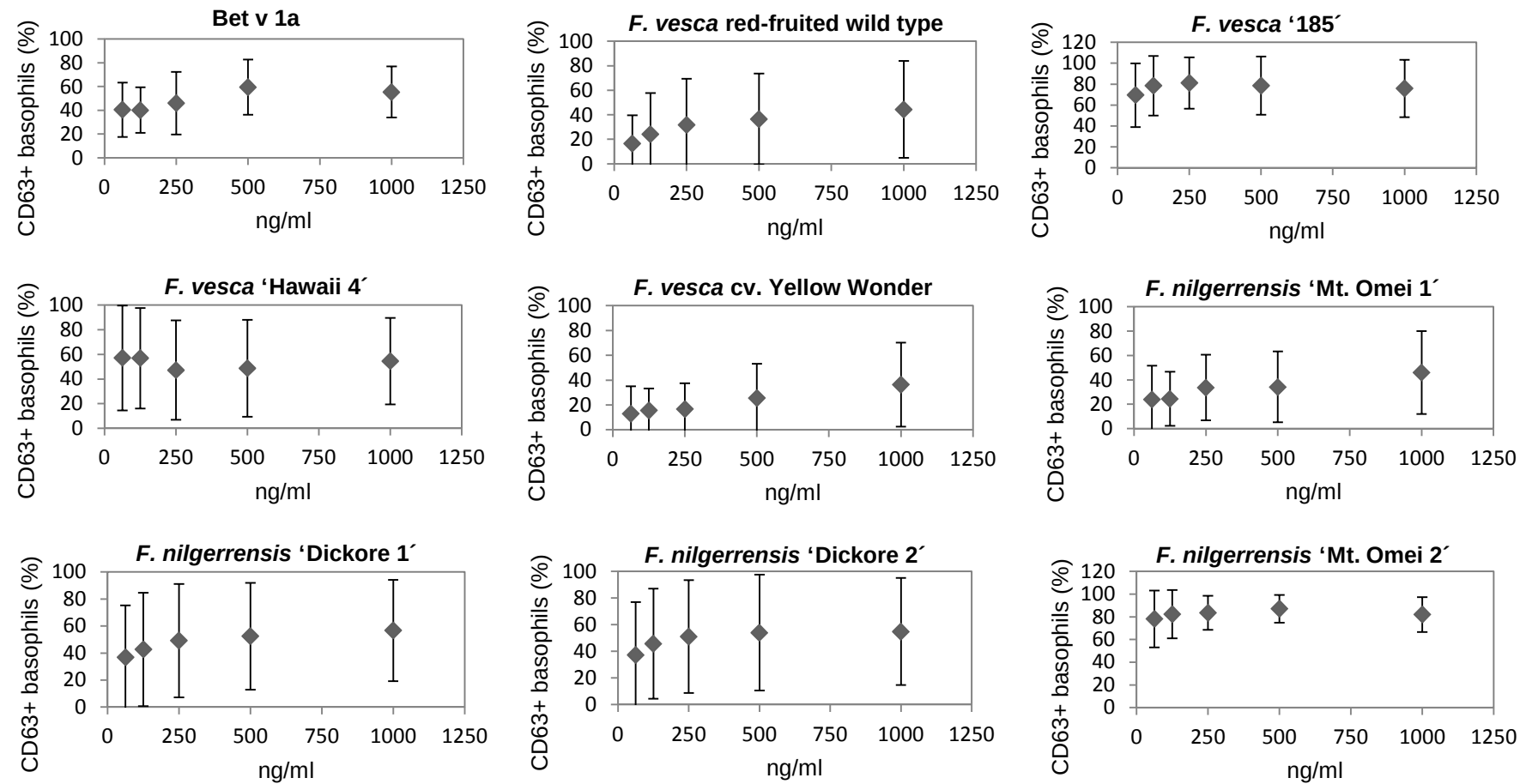


Figure S2a. Dose-response data for CD63+ expression in basophils (means $\pm$ SD) of birch pollen allergic patients (n = 8) after incubation with different concentrations of Bet v 1a and diverse protein extracts of *Fragaria* varieties.

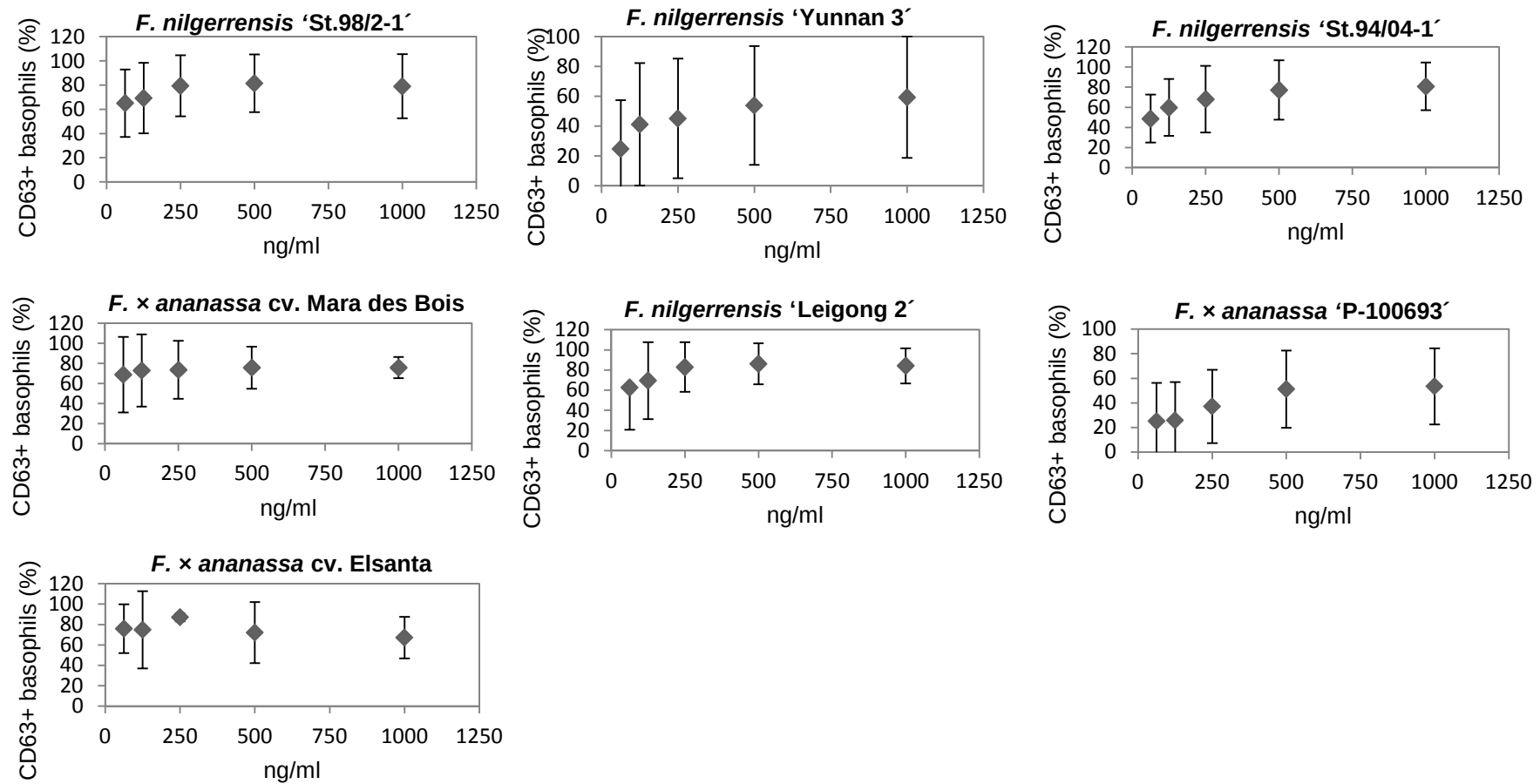


Figure S2b. Dose-response data for CD63+ expression in basophils (means $\pm$ SD) of birch pollen allergic patients (n = 8) after incubation with different concentrations of Bet v 1a and diverse protein extracts of *Fragaria* varieties.

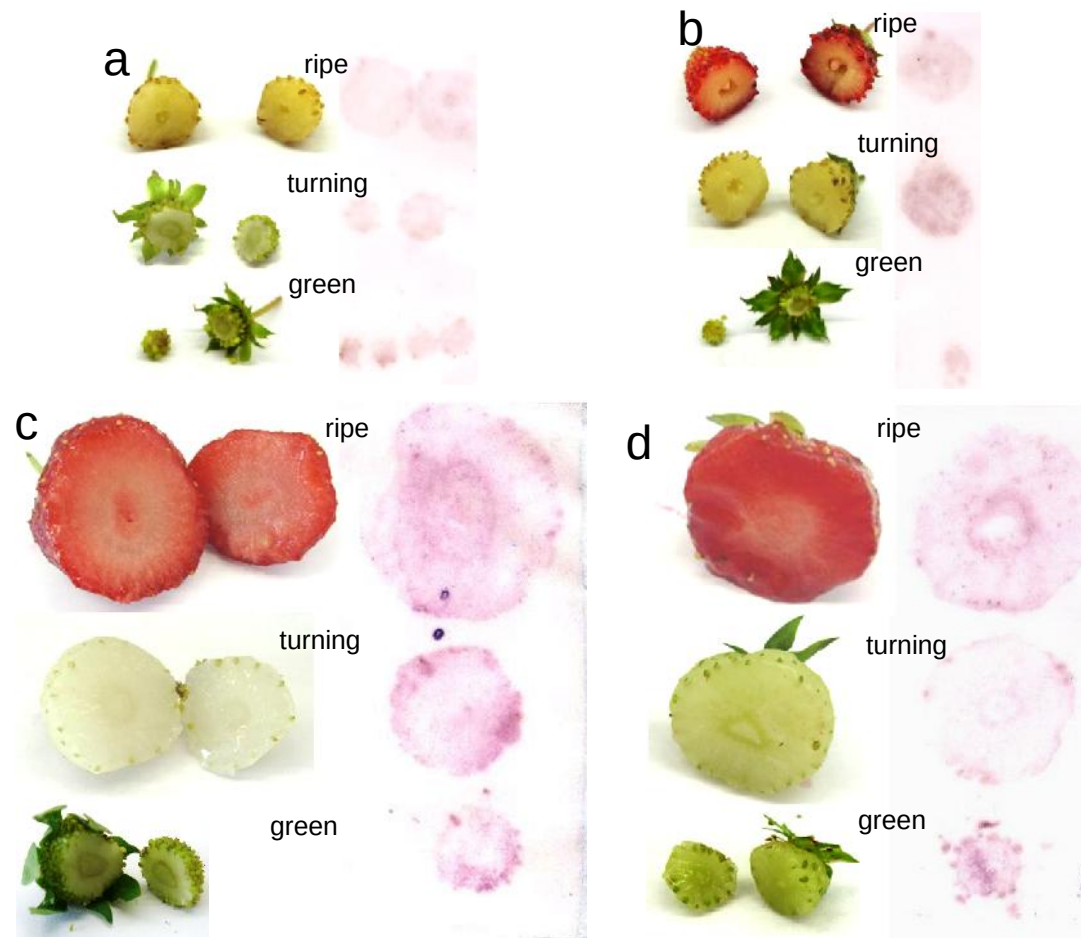


Figure S3. Allergen distribution in different ripening stages of cut strawberry fruits of different genotypes analysed by immuno tissue printing (ITP). Imprints of the strawberry fruit slices (vertical to core axis) were incubated with the polyclonal antibody raised against Fra a 1.02. (a) ITP of *F. vesca* cv. Yellow Wonder; (b) ITP of *F. vesca* red-fruited wild type; (c) ITP of *F. × ananassa* cv. Elsanta; (d) ITP of *F. × ananassa* cv. Mara des Bois.