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Breeding of hypoallergenic strawberry fruit[1]

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Abstract.
BACKGROUND: Allergy to food is a hypersensitivity disorder of the immune system to normally harmless food ingredients. A promising solution for allergenic patients is the development of hypoallergenic food.
OBJECTIVE: Selection and breeding of low-allergenic variety is the conventional strategy to produce hypoallergenic food. The strawberry fruit proteins Fra a 1.01E, Fra a 1.02 and Fra a 1.03 are homologous of the major birch pollen allergen Bet v1 but their individual allergenic potentials are unknown.
METHOD: We produced the recombinant Fra a allergens and evaluated their cross allergenic potential in birch pollen allergic patients by a basophil activation test. Anti-Fra a 1.02 antibodies were also used to screen for allergen deficient strawberry lines.
RESULTS: Although Fra a 1.01E, Fra a 1.02 and Fra a 1.03 have sequence similarities of 70, 71 and 74% with Bet v 1 Fra a 1.02 showed the highest allergenic potential. The data support the role of Fra a 1.02 as the major allergen for individuals affected by a strawberry allergy. The screening of strawberry varieties detected genotypes with significantly reduced levels of the allergen.
CONCLUSION: Genotypes with reduced Fra a 1.02 proteins might serve as starting material for the breeding of hypoallergenic strawberry varieties.

Keywords: Allergen, strawberry, Bet v 1, Fra a 1

1. Introduction

Allergy is an inappropriate reaction by the body's immune system to normally harmless substances such as proteins (allergens). Allergic reactions are distinctive because of excessive activation of certain white blood cells called mast cells and basophils by a type of antibody called Immunoglobulin E (IgE). This reaction which can be quantified by diagnostic test systems results in an inflammatory response which can range from skin reactions to anaphalytic shocks which can lead to death.

The occurrence of food allergies increases year to year, especially in industrial countries. In Europe, 2 – 4 % of the adult and 6–8% of the children population are affected by food allergy whereas 3.5 – 4 % of the adults in the US suffer from IgE mediated food allergy [1, 2]. Changes in life style including pattern of food consumption, hygiene, and child vaccination are some of the reasons thought to be responsible for the high prevalence. The simplest way to eliminate food allergy is the avoidance of the causing food. As many allergenic proteins are pan-allergens and can bind to IgE antibodies of different pollen and food allergenic patients, a number of foods have to be eliminated

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which is impossible, in particular for children. Alternatively, the development of hypoallergenic food is promising to improve life quality of allergenic patients. Such foods can be produced through the elimination or destruction of allergens by processing technologies such as chemical (acids), biochemical (proteases or peroxidases) and physical (heating or extraction) means or the generation of hypoallergenic primary materials by selection and breeding of new cultivars or by genetic engineering. The basic prerequisite of the methods is the identification of the true allergen as some of the proteins are encoded by gene families.

Fruits are one major source of food allergens. The resistance of some fruit proteins to proteolysis and their physicochemical properties appear to be key factors to induce direct sensitizations through the oral route, as well as systemic reactions upon ingestion [1, 2]. A number of allergens found in fruits belong to the group of pathogenesis-related (PR) proteins whose expression is induced in response to biotic and abiotic stresses. It is assumed that PR-10 proteins cause allergy only in people previously sensitized by epitopes of the birch major pollen allergen Bet v 1 as these proteins are degraded during the passage through the gastrointestinal tract.

Fruits from the rose family (*Rosaceae*) are widely consumed and have been reported as causes of hypersensitivity disorders which have been described frequently as a cross-reactive phenomenon linked to birch pollinosis. Allergenic reaction to strawberry (*Fragaria x ananassa*) is a commonly reported but poorly investigated [3].

In a search for hypoallergenic strawberry genotypes multiple proteins, homologous to Bet v 1 were detected in *F. x ananassa*. The total allergen content varied due to environmental conditions and was always lower in a white fruited strawberry variety than in red fruited genotypes. The ripe colorless fruits were tolerated by individuals affected by allergy as they were virtually free from the strawberry allergen. Isolation and sequencing of the Bet v 1 homologous allergen Fra a 1 from *F. x ananassa* revealed the presence of an intron and little variability in amino acid sequence (Fra a 1 isoforms A to E). However, the recent identification of additional Fra a genes and the complete genome sequence of the related species *F. vesca* show that the genus Fragaria contains a family of Fra a homologous genes [4–6].

To answer the question whether the expression of Fra a and color formation are related transient RNAi-mediated silencing of Fra a genes were performed in strawberry fruits of the red-fruited cultivar Elsanta [7]. Reduced levels of Fra a mRNAs caused significantly decreased levels of anthocyanins and upstream metabolites. These results demonstrate that the Fra a allergen has an essential biological function in pigment formation in strawberry fruit [4].

Sequence data from the *F. vesca* genome show that Fra a 1A – 1E [8] represent probably allelic forms. Thus, they were renamed as Fra a 1.01A – 1.01E, and Fra a 2 and 3 [4] as Fra a 1.02 and 1.03 in compliance with the nomenclature for allergen proteins. Although Fra a 1.01 has been described as the major allergens of strawberry fruit [8, 9] qPCR analyses demonstrated that only the Fra a 1.02 gene shows a ripening-related expression pattern [4]. Consequently, we studied the allergenic potential of the different Fra a 1 proteins in birch pollen allergic patients by a basophil activation test (BAT). The BAT is considered a valuable tool for the determination of sensitization to food and is routinely used for allergy diagnosis [10, 11]. Finally, the identification of the major strawberry allergen allowed the production of antibodies to screen different strawberry genotypes for their allergenic potential. The identification of suitable genotypes will facilitate the breeding of hypoallergenic strawberry cultivars with improved agronomic characteristics in the future.

2. Material and methods

2.1. Cloning of Fra a 1.01E, Fra a 1.02 and Fra a 1.03

The ORF sequence of three isoforms of the Fra a 1 PR10 protein (Fra a 1.01E, Fra a 1.02 and Fra a 1.03) were isolated and transformed in the *E. coli* strain BL21(DE3)pLysS (Novagen, Darmstadt, Germany) as previously described [4].

2.2. Heterologous expression and purification of recombinant Fra a 1 proteins

Production and purification of recombinant Fra a 1 proteins.were performed as recently described [12].

2.3. Basophil activation test by flow cytometry

The quantitative determination of rBet v 1a, and rFra a 1.01E/1.02/1.03-induced activation of human basophils by flow cytometry was conducted as previously reported [10, 11].

2.4. Screening for hypoallergenic genotypes

Proteins were extracted by phenol extraction [13] from ripe strawberry fruits of different white and red fruited Fragaria genotypes and separated by 12% separating SDS-PAGE under reducing conditions followed by silver or coomassie staining. Fra a 1 homologous proteins were visualized using polyclonal antibodies raised against Fra a 1.02.

3. Results

In total, 11 patients (2 males, 9 females) with a history of type-I-allergy to birch pollen and 3 controls (1 male, 2 females) were examined. A careful allergy history was taken, and skin tests and determination of specific IgE-antibodies were performed. Fra a 1.01E, Fra a 1.02, Fra a 1.03 and Bet v 1 were used at three different concentrations in a basophil activation test based on stimulation of whole blood cells measuring CD63 activation of basophils and using CCR3 as basophil marker by flow cytometry (Flow CAST®).

3.1. Basophil activation test

In patients with allergy to birch pollen Fra a 1.02 and Bet v 1 showed the highest activation of basophils. Whereas application of rBet v 1a and rFra a 1.02 resulted in the activation of appox. 50% of the basophils rFra a 1.01E and rFra a 1.03 activated less than 32.0%. Comparison of the mean basophil activation of the positive control rBet v 1a, the negative control pQE70 empty vector and the three rFra a 1 isoforms (rFra a 1.01E, rFra a 1.02 and rFra a 1.03) determined through BAT showed a significant difference between rFra a 1.02 and rFra a 1.01E and between rFra a 1.02 and rFra a 1.03. The results of the BAT clearly demonstrate that the isoform Fra a 1.02 is the major Bet v 1 homologous allergen in strawberry fruit.

3.2. Hypoallergenic strawberry fruits

To semi-quantify Fra a allergens in strawberry fruit, proteins were isolated from fruit tissue and separated by SDS-PAGE. Using polyclonal antibodies and Western blotting techniques the presence of Fra a 1 homologues were identified in variable amounts in different wild and cultivated strawberry varieties (Fig. 1). Two bands of 15 and 17 kDa were detected and confirmed by LC-MS as Fra a 1 homologous proteins. Up to now, only some of the white fruited genotypes (*F. vesca* 175 and *F. nilgerensis* 80) seem to be devoid of the allergen whereas red fruited Fragaria genotypes show at least one of the two Fra a 1 protein bands.

4. Discussion

4.1. Fra a 1.02 is the major allergen in strawberry fruit

We have analyzed three Bet v 1 homologous proteins that have been detected in ripe strawberry fruit [4, 9]. Although previous studies proposed isoforms of Fra a 1.01 as major strawberry allergens expression analyses indicated the accumulation of Fra a 1.02 transcripts during fruit ripening. Transient silencing of Fra 1.02 gene expression in strawberry fruits yielded ripe fruits that retained a white colour. Accordingly, Fra a 1.02 showed the highest allergenic potential of the three different Fra a isoforms in BAT. Although Fra a 1.01E, Fra a 1.02 and Fra a 1.03 have sequence similarities of 70, 71 and 74% with Bet v 1, the basophil activation of birch pollen allergic patients differed substantially

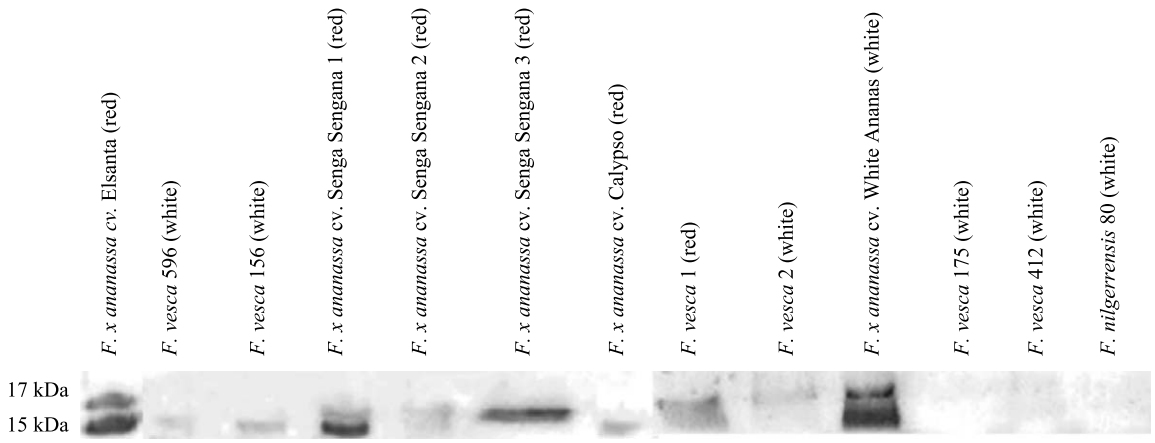

Fig. 1. Detection of Fra a proteins in different white and red fruited *Fragaria x ananassa* and *Fragaria vesca* genotypes by Western blot with polyclonal antibodies against Fra a 1.02.

between these isoforms. Fra a 1.01E and Fra a 1.03 revealed reduced capacities to activate basophils probably due to reduced IgE binding.

4.2. Screening for hypoallergenic strawberry fruit

Screening of numerous Fragaria genotypes for Fra a 1 proteins allowed the identification of some wild varieties that appeared devoid of the allergen. Thus, breeders can take advantage of the biodiversity of Fragaria to select for hypoallergenic strawberry lines. However, up to now only white fruited genotypes show reduced levels of the allergen. Remarkable, some of the varieties that produce white fruits accumulate even very high levels of Fra a 1 proteins (*F. x ananassa* cv. White Ananas). Thus, white fruited genotypes are not generally hypoallergenic as they probably harbour different mutations that cause the white phenotype.

5. Conclusions

The results demonstrate that small changes in the protein sequence can change orthologs in their allergenic characteristics although the overall 3D structure is almost identical. Our data also highlight the role of Fra a 1.02 as the major allergen for individuals affected by a strawberry allergy, because these patients tolerate white-fruited mutant genotypes. Screening for allergen-deficient strawberry fruits revealed hypoallergenic genotypes that can be used in future breeding programs.

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