RESEARCH ARTICLE

Peanut varieties with reduced Ara h 1 content indicating no reduced allergenicity

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Peanut allergy is a major cause of food-induced severe anaphylactic reactions. To date, no medical care is available to prevent and treat peanut allergy and therefore hypoallergenic peanut varieties are of considerable health political and economic interest. Major allergens that induce IgE-responses in peanut-sensitive patients are Ara h 1, Ara h 2 and Ara h 3/4. In order to identify hypoallergenic peanuts, commercially locally available peanut varieties were screened for their allergen content. Ara h 1-deficient peanuts from Southeast Asia were identified by SDS-PAGE, immunoblotting, inhibition assays and ELISA. 2-D PAGE analyses demonstrated the different compositions of the tested extracts and revealed a number of variations of the allergen patterns of peanuts from different varieties. Mediator release experiments of these peanut extracts demonstrated similar allergenicities as compared with standard peanut extract. These results indicate that the allergenicity of peanuts with reduced Ara h 1 content might be compensated by the other allergens, and thus do not necessarily cause a reduction of allergenicity.

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1 Introduction

Peanuts belong to the eleven most hazardous food allergens in the European Union. Since 2005, peanut as ingredient in pre-packaged foods must be declared according to the European food regulations [1] (http://europa.eu.int/eur lex/pri/en/oj/dat/2003/l_308/l_30820031125en0015-0018.pdf). This directive was introduced to minimize the accidental uptake of hidden food allergens. As shown by Hourihane *et al.* even minute amounts (as little as 100 μg) of peanut protein can elicit symptoms in peanut-sensitive patients [2]. As for other food allergies, there is no curative therapy available to treat peanut allergy. Therefore, in particular in the US, the idea emerged to generate peanuts with reduced allergenic potential by biotechnological strategies targeting the known major peanut allergens [3]. This approach is believed to increase the level of tolerated low-dose peanut uptake without eliciting severe symptoms and to reduce anaphylactic shock reactions by accidental ingestion of peanuts. However, this approach raises some questions regarding its feasibility, since our knowledge about the physiological function of the eleven identified allergens in peanut is still limited.

Is it possible to knock out allergens without destroying the durability and integrity of the peanut plant? Ara h 1 and Ara h 3/4 belong to the storage proteins of the 7S vicilin and 11S arachin type, respectively, and make up approximately 90% of the soluble peanut proteins. It is obvious that altering these proteins, which are encoded by multi gene families, might result in changes of the peanut in regard to

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Abbreviation: RBL, rat basophilic leukemia

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their agricultural performance such as yield. 2S peanut allergens of the conglutin type, *e.g.* Ara h 2, which is supposed to be trypsin inhibitor [4] may loose their protective influence with tremendous physiological consequences.

Since the immune system of peanut-sensitive patients defines the allergenic proteins in this legume, we must determine its reactions to the altered peanut. Including functional tests, recombinant approaches to produce hypoallergenic peanuts may result in lower IgE reactivity. But the immune system is a dynamic adaptable system, which can be misguided. Therefore, it might be a more straightforward approach to search for hypoallergenic peanut varieties by analyzing varieties from different geographic locations. If this search were successful, this would have the advantage to study the effect of already existing hypoallergenic peanut varieties on the phenotype of the plant and agricultural performance and, more importantly, on the immune system of their consumers.

With the first approach we investigated consumed peanut varieties from Southeast Asia and observed especially in varieties from Bali alterations in the protein pattern. We screened by Western blotting and determined differences in the protein content of extracts from standard and Balinesian variants by high-resolution 2-D gel electrophoresis. The identified differences were further studied by *in vitro* assays for their effects on the immune system of peanut-sensitive patients.

2 Materials and methods

2.1 Sera and antibodies

Patients included in this study are sensitized to peanut according to case history. Sera of five patients (JH, SK26, YW, PS42, WZ) were collected at the Research Center Borstel (Borstel, Germany) and were specified elsewhere [5, 6].

The following polyclonal and monoclonal antibodies were used: rabbit anti-Ara h 1 (KB), mAb anti-Ara h 1 (Pn-t and Pn-c (the precise epitopes on the Ara h 1 could not be determined by pepscanning, however, the mAb belong the IgG1 and IgG2b subclasses, respectively)), mAb anti-Ara h 2 (Pn–2) and mAb anti-Ara h 3/4 (Pn-x) [7].

2.2 Extraction of peanut proteins

Fourteen peanut varieties were purchased in Southeast Asia from suppliers (Kacang Asin from Manalagi in Denpasar, Bali, Indonesia, called ''Bali-1''; Kacang Kulit from Garudafood, Jakarta, Indonesia, called ''Bali-2'') and from local supermarkets (termed according to their regional provenance ''Indonesia'' and ''Thailand''). The peanuts purchased in Germany (Seeberger, Ulm) were of the Virginia type and defined as standard peanut in this study. The peanut kernels were extracted overnight in 0.1 M NH_4HCO_3, pH 8.0 as previously described [7]. The extracts were dialyzed against *aqua bidest.* overnight, lyophilized and stored at −20°C.

2.3 Purification of Ara h 1

Ara h 1 was purified from defatted standard peanut extract by concanavalin A (Con A)-affinity chromatography [8]. Purified Ara h 1 showed a major band of 63 kDa by SDS-PAGE. The Ara h 1 content was determined at 280 nm using the molar extinction coefficient. An absorbance of $A_{280} = 1.75$ is equivalent to 1 mg/mL Ara h 1.

2.4 SDS-PAGE and immunoblotting

SDS-PAGE was performed according to Laemmli [9] using the PerfectBlue Dual System (Peqlab Biotechnology, Erlangen, Germany) with 12% acrylamide gels. Afterwards, gels were stained with CBB R-250.

For immunoblot analysis, the separated proteins were subsequently transferred to PVDF membranes (Millipore, Schwalbach, Germany) by semi-dry blotting for 30 min at 0.8 mA/cm^2 [10]. The membranes were blocked for 2 h in TTBS (100 mM Tris, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.05% v/v Tween 20, pH 7.4) containing 5% w/v non-fat dry milk. Incubations of membranes with polyclonal antiserum, monoclonal antibodies or patients' sera, diluted in TTBS, were carried out overnight. Bound antibodies were detected with AP-conjugated secondary antibodies (goat anti-rabbit IgG-AP (Dianova, Hamburg, Germany), goat anti-mouse IgG/IgM-AP (Dianova), anti-human IgE-AP (Allergopharma, Reinbek, Germany) for 2 h. The staining reaction was performed using the NBT/BCIP system [11].

2.5 2-D gel electophoresis (2-D PAGE)

Lyophilized peanut extracts were dissolved on ice for at least 30 min with an appropriate volume of IEF solubilization mix (7 M urea, 2 M thiourea, 30 mM Tris, 4% w/v CHAPS pH 8.5) to adjust the protein concentration to 5 mg/mL. 100 µg of the protein with a concentration of 2 µg/µL were diluted with 50 µL rehydration buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 2% v/v IPG buffer pH 3–11 non-linear and 2% w/v DTT) and applied onto 18 cm IPG non-linear pH 3–11 gel strips (GE Healthcare, Munich, Germany) by cup loading. The gel strips were rehydrated at ambient temperature for an overnight period in 340 µL DeStreak solution (GE Healthcare) with 0.5% v/v IPG buffer pH 3–11 non-linear (GE Healthcare). IEF was carried out with 50 µA *per* strip at 20°C for a total of 51750 Vh in five steps with a gradual increase of voltage (150 V for 3 h, 300 V for 3 h, gradient from 300 to 1000 V for 6 h, gradient from 1000 to 10 000 V for 3 h and 10 000 V for 3 h, $\Sigma = 18$ h) using the IPGphor 3 system (GE Healthcare). For the second

dimension the IPG strips were first reduced for 15 min in SDS equilibration buffer (6 M urea, 50 mM Tris-HCl, pH 8.8, 30% v/v glycerol, 2% w/v SDS, a trace of bromphenol blue and 0.5% w/v DTT) and then carbamidomethylated for 15 min in the same buffer containing 4.5% w/v iodacetamide instead of DTT. The second dimension was performed at ambient temperature on 14% polyacrylamide gels (16 × 20 cm) using the PROTEAN II xi system (Bio-Rad, Munich, Germany) at 60 V until the bromphenol blue band reached the end of the gel. The silver-stained gels were scanned on the molecular imager GS-800 (Bio-Rad) and compared with each other by visual inspection.

2.6 Sandwich-ELISA for quantification of Ara h 1

Microtiter plates were coated with 18 ng/well of capture monoclonal antibody Pn-t (anti-Ara h 1) in 0.3% w/v sodium acetate solution, pH 5.0, overnight at 4°C. Subsequently, wells were washed seven times with TTBS (TBS containing 0.05% v/v Tween 20) and blocked for 2 h with 3% w/v milk powder in TTBS to reduce non-specific binding. Plates were incubated with purified Ara h 1 as standard, and with conventional and Bali-1 peanut extract in serial dilutions from 0.1 pg/mL to 0.1 mg/mL for 2 h in assay buffer (1.5% w/v milk powder in TTBS). After washing, bound Ara h 1 was detected by using rabbit anti-Ara h 1 serum KB (1:1000 dilution and incubation for 2 h) followed by AP-conjugated anti-rabbit monoclonal antibody (1:5000 dilution and incubation for 2 h). The determination was performed by use of pNPP as substrate. The enzymatic reaction was stopped after 30 min with 3 N NaOH. The adsorption was determined at 450 nm. The Ara h 1 contents in the different extracts were determined based on the parameters of a four-parameter logistic non-linear regression analysis.

2.7 Mediator release assay

The mediator release assay of rat basophilic leukemia (RBL) cells was performed according to Hoffmann *et al.* [12] with some modifications. The humanized RBL cell subline of the RBL-2H3 named RBL-203/75 (a generous gift from Dr. Stefan Vieths, Paul-Ehrlich-Institute, Langen, Germany) was maintained in RBL-medium (80% v/v minimal essential medium, 20% v/v RPMI-1640, supplemented with 5% v/v fetal calf serum, 1% v/v L-glutamine and 0.001% v/v geneticine sulphate) in a humidified atmosphere of 5% CO_2/95% air [13]. Cells were incubated for 2 h at 37°C to attach to the surface of a microtiter plate (Nunc, Wiesbaden, Germany) (1×10^5 cells *per* well). Passive sensitization of the cells was performed overnight by incubation with patients' sera in a final dilution of 1:20 in RBL-medium. Afterwards, cells were washed in Tyrode's buffer (130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 10 mM HEPES and 0.1% v/v bovine serum albumin, pH 7.4). For cross-linking reaction, allergens were added to the wells, prediluted in Tyrode's buffer in serial dilutions. Negative controls were run without allergen to calculate the spontaneous release and positive controls with Tyrode's buffer containing 1% Triton X-100 (Sigma, Deisenhofen, Germany) to quantify the total release. After incubation, the enzymatic activity was detected by transferring of 20 µL supernatant to a second 96-well plate, containing 50 µL *p*-nitrophenyl *N*-acetyl β-D-glucosaminide (1.3 mg/mL in 0.1 M citric buffer, pH 4.5). The reaction was stopped after further incubation for 30 min. The adsorption was read at 405 nm. Results were calculated as percentage of the total release after subtraction of the spontaneous release and were plotted on an *x*–*y* graph by log *x* scale. The biological activity of the different extracts was calculated by the parallel line method and compared with the standard peanut extract by using the 30% release value.

3 Results

3.1 Characterization of peanut allergens by SDS-PAGE and immunoblotting

Peanuts of different varieties were extracted and analyzed by SDS-PAGE. Figure 1 shows the results of gel electrophoresis of the standard peanut extract and various peanut extracts from Southeast Asia: three varieties from Indonesia (Indonesia, Bali-1 and Bali-2) and one from Thailand. Differences were observed in the patterns and the extractable amount of proteins. Most notable, a band of approximately 63 kDa was missing in both Bali peanut extracts (Bali-1 and Bali-2). Performing immunoblot analysis using two Ara h 1-specific monoclonal antibodies (Pn-t and Pn-c), we detected that Ara h 1 was lacking in the extracts of Bali peanut (data not shown). Immunoblot of standard peanut extract and inhibition of the monoclonal anti-Ara h 1 antibodies by preincubation with Bali-1 and Bali-2 peanut extract caused only a partial absorption of these antibodies,

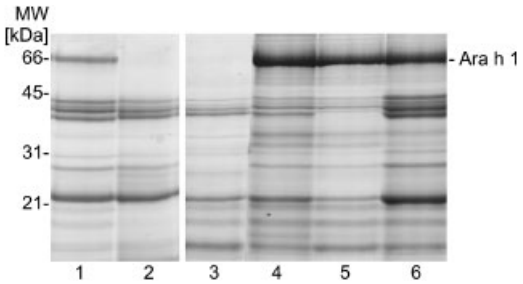

Figure 1. Analysis of different peanut extracts by SDS-PAGE. In total 15 µg extract/cm were run on a 12% SDS-PAGE gel under reducing conditions, Coomassie staining. Lane 1: standard peanut extract, lane 2: Bali-1 peanut extract, lane 3: Bali-2 peanut extract, lane 4: Thailand peanut extract, lane 5: Indonesian peanut extract and lane 6: standard peanut extract.

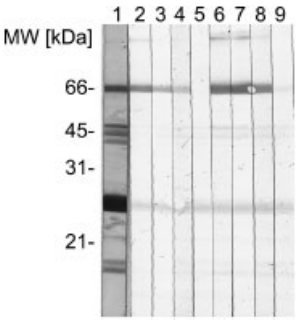

Figure 2. Analysis of Ara h 1 content in peanut extracts by inhibition assay. Western blot of standard peanut extract (15 μg/cm) on PVDF membrane. Inhibition of the primary Ara h 1 monoclonal antibody (Pn-t or Pn-c) with Bali-1 and Bali-2 peanut extract. Lane 1: India Ink staining; lane 2: Pn-t without inhibition; lanes 3, 4, 5: Pn-t preincubated with Bali-1, Bali-2 and standard peanut extract, respectively; lane 6: Pn-c without inhibition; lanes 7, 8, 9: Pn-c preincubated with Bali-1, Bali-2 and standard peanut extract, respectively.

compared with Ara h 1 containing standard peanut extract (Fig. 2). Inhibition of Pn-t by Bali-1 and -2 peanut extract showed a slight absorption of the monoclonal antibody, indicating only traces of Ara h 1 in these extracts. However, a preincubation of the monoclonal antibody Pn-c with both Bali peanut extracts did not result in any inhibition.

3.2 Quantification of Ara h 1 in peanut extract

Since the protein patterns of various peanut varieties showed significant differences in the Ara h 1 content by SDS-PAGE and immunoblot analysis, a sandwich ELISA was developed for the quantification of the Ara h 1 amount in the Bali-1 and standard peanut extract. A standard curve was generated using Ara h 1 in serial dilutions. Figure 3 shows that the curve of similar dilutions of standard peanut extract is parallel to the standard curve of Ara h 1. Comparing the inflection points, crude peanut extract contains 32% of Ara h 1. Ara h 1 in Bali-1 peanut extract is nearly undetectable. Even after applying higher concentrations of Ara h 1, the absorption did not exceed OD_{405} of 0.7, which suggests an Ara h 1 content of less than 0.2% in the Bali-1 peanut extract.

3.3 2-D analysis of peanut protein

To visualize the obvious differences of extracts from standard peanuts (Fig. 4A) and Balinesian variants in high resolution, we analyzed the protein content composition by 2-D PAGE. The identification of the different allergens was performed by immunoblotting with group specific monoclonal antibodies (Pn-c = anti-Ara h 1, Pn-t = anti-Ara h 1, Pn-2 = anti-Ara h 2, Pn-x = anti-Ara h 3/4) (data not shown). As shown in Fig. 4, major differences between the two varieties can be summarized as follows: extracts of Balinesian peanuts

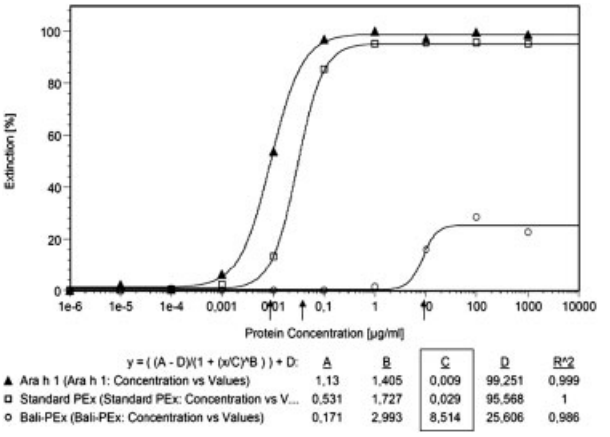

Figure 3. Quantification of Ara h 1 in standard and Bali-1 peanut extract by Sandwich-ELISA. The monoclonal anti-Ara h 1 antibody Pn-t was coated on a microtiter plate (0.3 μg/mL). Purified Ara h 1 (circles), standard peanut extract (squares) and Bali-1 peanut extract (triangles) were used in serial dilutions to determine the Ara h 1 amount. Detection was performed with polyclonal rabbit anti-Ara h 1 and alkaline phosphatase conjugated anti-rabbit antibody. The curves and the protein concentrations were determined by the equation below the diagram. A, the upper plateau of the curve; B, the inclination, C, the effective concentration at 50%; D, the lower plateau and R^2, the coefficient of correlation.

(Fig. 4B) lack the major allergen Ara h 1 (a) almost completely, the major allergen Ara h 2 (b) is strongly reduced. Some proteins (d, e) can be detected in extracts from the Balinesian variant, but they are not detectable in standard peanuts.

3.4 Biological activity

To compare the biological activity of the Bali-1 peanut extract with standard peanut extract, the RBL mediator release assay was performed. After passive sensitization of RBL cells with IgE of peanut-allergic patients, the cells are challenged by serial dilutions of standard and Bali-1 peanut extracts. Figure 5 shows that the release of the five sera depends on the protein concentration of the different extracts. The resulting dose-related mediator release plots are nearly identical for standard and Bali-1 peanut extracts indicating that they have the same biological potency. For a direct comparison of both extracts, the protein concentration was compared, which is needed to obtain a value of 30% of total release. There is no decrease in the allergenicity of the peanut variety, which lacks the major allergen Ara h 1. The patients' sera JH and SK26 showed even a slight, but not significant increase in mediator release after addition of Bali-1 peanut extract, whereas YW shows a two times and WZ a ten times increased allergenicity of the Bali-1 peanut in comparison to the standard peanut. Only PS42 indicates a slight, but not significant decrease in the allergenicity of the Bali-1 peanut. The IgE-reactivity of patients' sera was characterized by immunoblot analysis. All patients' sera detected

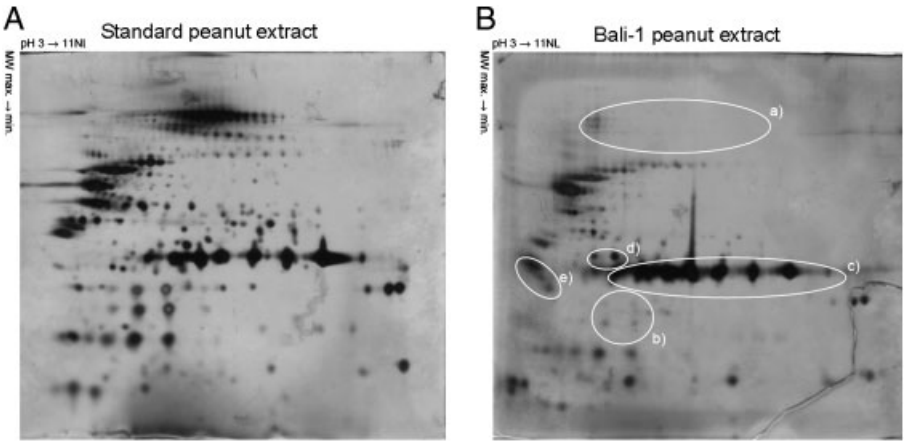

Figure 4. Comparison of extracts from standard (A) and Balinesian peanuts (B) by 2-D PAGE. First dimension: IEF (pH-range 3–11, non-linear, 18 cm), second dimension: 14% SDS-PAGE gel, reducing conditions. Proteins were visualized by silver staining. The following positions are circled: (a) Ara h 1; (b) Ara h 2; (c) Ara h 3/4; (d) and (e) differentially expressed proteins with higher expression rates in Balinesian peanuts. (The gels were run in triplicate; one representative gel is shown.)

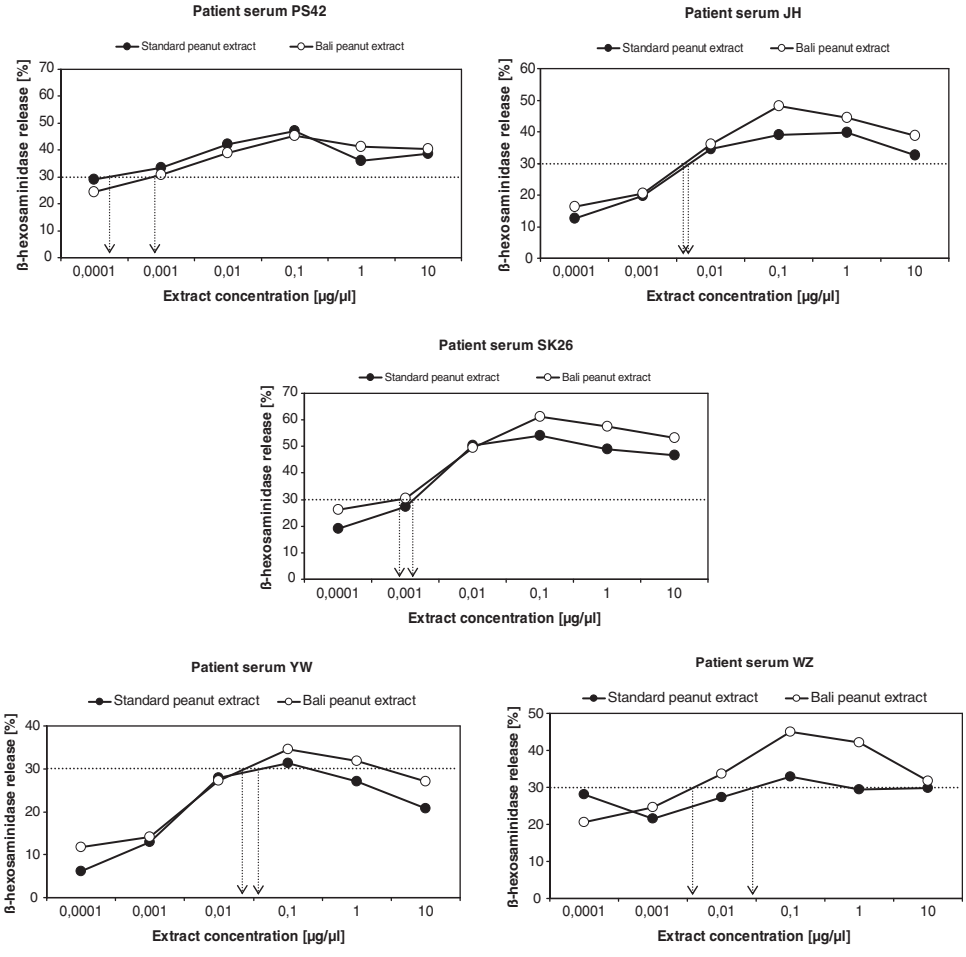

Figure 5. Cross-reactivity between extracts from standard and Bali-1 peanut extract. RBL cells were sensitized with IgE from peanut allergic patients. Degranulation was measured by β-hexosaminidase release after stimulation with the two different extracts. Dotted lines indicate the protein concentrations of standard and Bali-1 peanut extract causing 30% histamine release.

the same allergen pattern including the major allergens Ara h 1, Ara h 2 and Ara h 3/4 (data not shown).

4 Discussion

Peanut allergy is one of the most life-threatening food allergies. The number of peanut-allergic patients is still increasing mostly due to the ubiquitous use of peanuts as ingredient in processed foods. Currently, there are no curative therapies available for peanut allergy. To decrease the allergenicity of peanuts, one approach was to reduce one of the three major allergens, Ara h 2, through RNA interference [3]. In our study, we identified natural peanut varieties from Indonesia with a reduced content of the major allergen Ara h 1. This is in accordance with the

results of Schmidt *et al.* [14], who performed a detailed investigation of the protein patterns of both peanut varieties treated under various extraction conditions. Their results clearly showed a dramatic reduction of Ara h 1 in the Bali peanuts.

Indonesia has a great variety of regional peanut cultivars. This might be explained by the history of peanut cultivation and the early introduction of peanut cultivation to Indonesia. Peanut originating from South America was brought to the Philippines and then to Indonesia during the 16th century. Later the peanuts were introduced to North America *via* the detour to Africa in the 19th century. Today, China, the US and Indonesia belong to the top-ranking producers of peanuts, while the export from Indonesia is very low. To obtain peanut seeds from different ''exotic'' cultivars, we bought them in Indonesia from different producers and markets.

To analyze peanut extract of different cultivars, we performed SDS-PAGE and immunoblot analyses. Two peanut varieties were identified with decreased content of the major allergen Ara h 1. To quantify the Ara h 1 content, an Ara h 1-specific sandwich ELISA assay was developed. Commercially available assays are based on the detection of total peanut protein by use of rabbit antisera [15], but the two peanut varieties lacking Ara h 1 and such specific assays were not suitable. We purified Ara h 1 from peanut extract and used it as a standard protein in the assay. A sandwich ELISA was established with the monoclonal Ara h 1 antibody Pn-t as capture antibody and a polyclonal rabbit antiserum raised against Ara h 1 as detection antibody. This assay has a detection limit of 10 ng Ara h 1 *per* mL. The sensitivity was three times higher than the sandwich-ELISA developed by Pomes *et al.* [16], which is based on two monoclonal antibodies. Peanuts normally have a total protein content of 25% [17]. In this work the Ara h 1 content in the standard peanut was determined to be 7.8%. This confirms the results of Koppelman *et al.* [17], who analyzed the Ara h 1 content with biochemical methods to be 12–16%, whereas Pomes *et al.* detected only 0.05–1.5% Ara h 1 in the total protein extract. It was not possible to determine the exact Ara h 1 content in the Bali peanut indicating only traces of this allergen.

We hypothesized that the reduced content of the major allergen Ara h 1 correlates with a reduced allergenicity. To confirm this assumption, an RBL-assay was performed. Interestingly, using this functional biological assay the reactivity of the Bali peanut extract was nearly identical to that of standard peanut extracts. These results are in line with the observation that Indonesia is the country with the highest rate of peanut allergy in Southeast Asia [18]. Consequently, the lack of the major allergen Ara h 1 in peanuts does not seem to have an effect on the Indonesian population.

Further studies should be performed to analyze the genomic and expression status of Ara h 1 in Ara h 1-deficient peanut varieties. The question arises, whether genetic defects and/or down-regulation of mRNA expression is responsible.

It remains still unclear whether such hypoallergenic peanuts obtained from transgenic peanut plants would be tolerated by patients allergic to peanuts, because even a small amount of residual peanut allergens or the increased expression of the other allergens still present could elicit allergic reactions. Therefore, a knock out of single allergens must be considered critically. The strategy should not only be to down regulate an allergen but also to exclude probable changes of the other allergens that are still present.

Peanuts contain at least eleven different allergens, but little is known of their physiological function, with the exception of their role as storage proteins. The effect of specific knockouts on the agricultural performance, and physiology of plant and seed cannot be predicted in advance. Does the plant tolerate the loss of single storage proteins? Will the taste of the peanut kernels be influenced? Some allergens are members of a multigen family or occur in high gene copy numbers. Thus, at least for those allergens the knockout approach seems to be unsuitable. In this study, we presented a peanut variety with one naturally down-regulated major allergen, but with similar allergenicity, which might be due to the compensation by other allergens of the same function (*e.g.* seed storage proteins).

The authors have declared no conflict of interest.

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