

Alleviating peanut allergy using genetic engineering: the silencing of the immunodominant allergen Ara h 2 leads to its significant reduction and a decrease in peanut allergenicity

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Summary

Peanut allergy is one of the most life-threatening food allergies and one of the serious challenges facing the peanut and food industries. Current proposed solutions focus primarily on ways to alter the immune system of patients allergic to peanut. However, with the advent of genetic engineering novel strategies can be proposed to solve the problem of peanut allergy from the source. The objectives of this study were to eliminate the immunodominant Ara h 2 protein from transgenic peanut using RNA interference (RNAi), and to evaluate the allergenicity of resulting transgenic peanut seeds. A 265-bp-long PCR product was generated from the coding region of Ara h 2 genomic DNA, and cloned as inverted repeats in pHANNIBAL, an RNAi-inducing plant transformation vector. The Ara h 2-specific RNAi transformation cassette was subcloned into a binary pART27 vector to construct plasmid pDK28. Transgenic peanuts were produced by infecting peanut hypocotyl explants with *Agrobacterium tumefaciens* EHA 105 harbouring the pDK28 construct. A total of 59 kanamycin-resistant peanut plants were regenerated with phenotype and growth rates comparable to wild type. PCR and Southern analyses revealed that 44% of plants stably integrated the transgene. Sandwich ELISA performed using Ara h 2-mAbs revealed a significant ($P < 0.05$) reduction in Ara h 2 content in several transgenic seeds. Western immunoblotting performed with Ara h 2-mAb corroborated the results obtained with ELISA and showed absence of the Ara h 2 protein from crude extracts of several transgenic seeds of the T₀ plants. The allergenicity of transgenic peanut seeds expressed as IgE binding capacity was evaluated by ELISA using sera of patients allergic to peanut. The data showed a significant decrease in the IgE binding capacity of selected transgenic seeds compared to wild type, hence, demonstrating the feasibility of alleviating peanut allergy using the RNAi technology.

Keywords: peanut allergy, allergen Ara h 2, gene silencing, hypoallergenic peanut, transgenic peanut.

Introduction

Modern biotechnology has brought over the past 15–20 years major changes in fields as varied as pharmaceuticals, medical diagnostics, forensic sciences, agriculture, and food production. Genetic engineering offers novel solutions impossible to imagine just few years ago, that can now be exploited

to provide realistic solutions to age-old problems as reported in several articles (Horn *et al.*, 1995; Clark *et al.*, 2002; Hulse, 2004; World Health Organization, 2005) and possibly peanut allergy (Dodo *et al.*, 2005). Peanut allergy is the most common cause of severe or fatal food-associated anaphylaxis and results in approximately 200 deaths per year in the USA alone (Bock *et al.*, 2001). It was estimated that 1.1% of the American

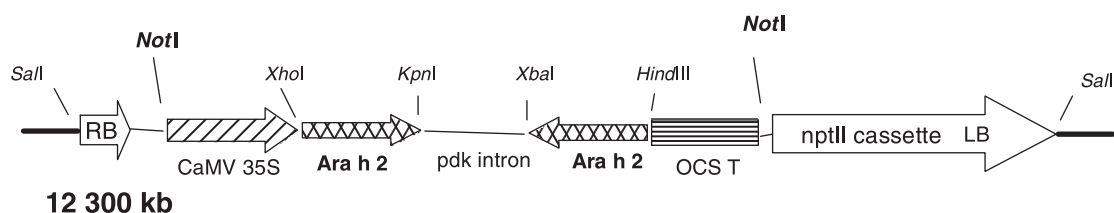


Figure 1 Schematic representation of the binary vector pDK28. The Ara h 2 transgene fragment was first mounted in inverted repeats separated by the pdk intron in pHANNIBAL. The transformation cassette was then subcloned into the binary vector pART27 as a *NotI* fragment

population or about 4 million people suffer from allergy to peanut and tree nuts (Sicherer *et al.*, 2003). In the USA, the prevalence of peanut allergy has doubled among children in the 5 years from 1997 to 2002 (Sicherer *et al.*, 2003). Peanut allergy is an abnormal response of the immune system to a particular food component, usually a naturally occurring protein that is not harmful to the body. It is a hypersensitivity reaction mediated by immunoglobulin E (IgE) (Taylor, 1992), which is acute with severe symptoms, life threatening, and outgrown by only few, unlike allergy to egg and cow's milk (Bock, 1982). The number of deaths due to accidental ingestion of peanut is increasing mostly due to its ubiquitous use and its undeclared and hidden presence in many food products.

Yet to date, there is no cure for peanut allergy. The most effective management option available to the susceptible population is assiduous avoidance of peanuts and peanut-containing products. However, avoiding peanut has proven difficult, particularly due to undeclared peanut in food. For example, at least 75% of patients with peanut allergy still failed to avoid peanut in food products even if they were carefully trying to do so (Weisnagel, 2002; <http://www.allerg.qc.ca/peanutallergy.htm>). Epinephrine and antihistamines are administered only to alleviate the symptoms (Berg, 2004).

The increasing number of news headlines around the world about peanut allergy, its negative impact on the peanut and food industries, and its significant health consequences demonstrate the dire need to find a cure to this problem.

Many efforts are being deployed to combat peanut allergy (Oppenheimer *et al.*, 1992; Nelson *et al.*, 1997; Roy *et al.*, 1999; Maleki *et al.*, 2000; Beyer *et al.*, 2001; Leung *et al.*, 2003), and several of them deal with ways to alter the immune system of patients allergic to peanut including immunotherapy, DNA and protein vaccines (Berg, 2004). Alternatively, we are using the RNAi technology to alleviate peanut allergy by eliminating the culprit, the allergenic proteins. The resulting hypoallergenic peanut is expected to save lives as it would trigger no reaction or at the most a mild one upon accidental ingestion of peanut. RNAi is a widespread naturally occurring phenomenon which offer the most efficient strategy for

customized down-regulation or silencing of gene expression (Matzeke *et al.*, 2001; Tang and Galili, 2004) resulting in the suppression or elimination of targeted proteins.

To demonstrate proof of concept in reducing the risks of peanut allergy via RNAi, we selected to silence Ara h 2, the most immunodominant allergen causing over 85% allergic reactions and with over 52.5-fold more potency than Ara h 1 (Koppelman *et al.*, 2001, 2004). Ara h 2 is a 17.5-kDa glycoprotein with an isoelectric point of 5.2. (Burks *et al.*, 1992). A cDNA sequence for Ara h 2 was published (Burks *et al.*, 1995), and the first genomic clone was isolated and sequenced in our laboratory (Viquez *et al.*, 2001). It is a full-length clone with an open reading frame of 622 nucleotides containing no intron, a deduced amino acid of 207 residues, 10 antigenic sites (IgE binding) with three of them characterized as immunodominant (Stanley *et al.*, 1997). The objectives of this study were to silence the gene encoding Ara h 2 using RNAi and to assess the allergenic potency of the resulting transgenic peanuts.

Results

Regeneration of fertile transgenic peanut

We previously reported the production of several transgenic peanut plants expressing a truncated Ara h 2 transgene (Konan *et al.*, 2004). However, upon maturity, the plants were sterile and no seed was produced, preventing the assessment of the silencing of Ara h 2 in transgenic peanuts. To circumvent this problem, an *Agrobacterium*-mediated transformation method was adopted. A total of 555 hypocotyl explants from the peanut Runner type Georgia green were infected and cocultured for 5 days with *A. tumefaciens* EHA105 harbouring the transformation vector pDK28 (Figure 1). Fertile transgenic peanut plants were produced within 5–7 months. The key steps involved in the production of fertile transgenic peanuts are shown in Figure 2. The percentage of regeneration of infected hypocotyl explants was significantly influenced by the preselection period. Explants transferred

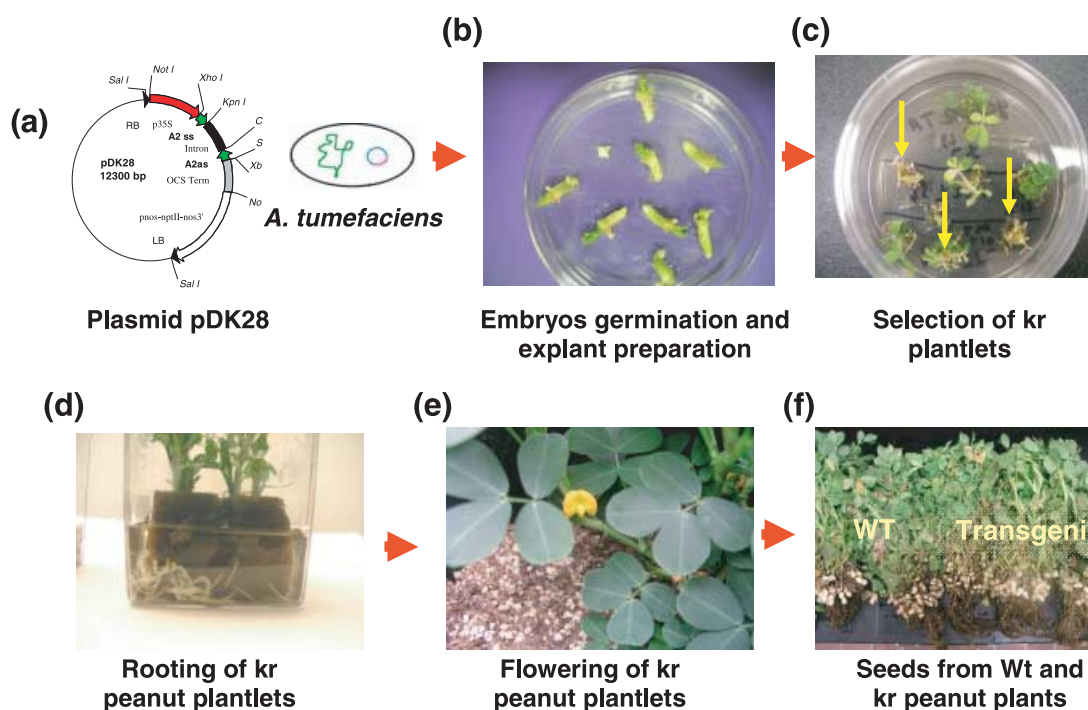


Figure 2 Steps involved in the production of fertile transgenic peanut plants using pDK28, an Ara h 2 RNAi transformation vector. (a) Map of pDK28 binary plasmid mobilized to *Agrobacterium tumefaciens* EHA105. (b) Germinated peanut embryos for hypocotyl explants preparation and co-cultured. (c) Selection of kanamycin-resistant (Kr) shoots. (d) Rooting of regenerated Kr plants. (e) Transfer to soil and flowering. (f) Seeds from Kr peanut plants.

on to the selection medium immediately after the coculture period (preselection = 0 day) died, and only 2 out 240 (0.8%) produced shoots among which 2 kanamycin-resistant plants were recovered. However, if the application of the selection pressure after the coculture period was delayed, a significant increase in the regeneration and number of kanamycin-resistant plants was observed. Explants transferred on the selection medium 1 week after the coculture period (preselection = 1 week) produced 11.5% (22/190) regenerants, and those transferred with 3 weeks' delay (preselection = 3 weeks) produced 18.4% (23/125) regenerants. In the later case, shoots reaching about 1 cm height were excised and transferred on to the selection medium. The number of shoots of 0.5–1 cm long varied between 1 and 3 per regenerating explant. Excised shoots were individually cultured on the selection medium for 6 weeks with medium refreshment after the first 3 weeks. Kanamycin-sensitive plants turned white and died within 4 weeks, while kanamycin-resistant plants remained green and grew healthy (Figure 2d). A total of 59 kanamycin-resistant plants (Ara h 2- T_0) were successfully rooted (Figure 2e) and transferred to pots (Figure 2f) in the Enconair growth chamber.

Flowering occurred within 6 weeks following the transfer to the growth chamber (Figure 2g). Flowers were produced on both controls obtained by (i) direct peanut seed planting

in soil (wild type) and (ii) tissue culture without *Agrobacterium* infection. The phenotype, plant growth and reproduction of the putative transgenic plants were indistinguishable from that of wild-type control. Peg elongated from the plants, and peanut pods were produced within 3–4 months (Figure 2h). The control wild-type plant obtained from direct seed planting in soil produced 25 mature pods, while the tissue culture-derived control plant produced 7 mature pods. The number of mature pods collected from each kanamycin-resistant plant could be grouped as follows: 11 plants produced between 2 and 5 pods, 14 plants produced between 6 and 10 pods, 17 plants produced between 11 and 20 pods, and 17 plants produced more than 20 pods with a maximum for plant #9, #52 and #55 which produced 32, 31 and 31 pods, respectively. Pods from both controls and kanamycin-resistant plants contained either 1 or 2 seeds as is typical for Georgia green a Runner market type.

PCR and Southern hybridization confirmed the stable transformation of kanamycin-resistant peanut plants

PCR analyses were performed to screen kanamycin-resistant plants for the presence of the transgene. Primers were designed to amplify both the CaMV 35S promoter and the *NPTII* selection marker gene present in the pDK28 transformation

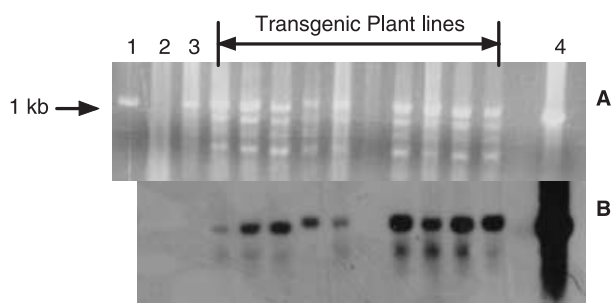


Figure 3 Molecular screening of transgenic T_0 (first generation) peanut plants. (a) Polymerase chain reactions (PCR) using primers specific to the CaMV 35S promoter. PCR products were resolved on 0.8% agarose gels. (b) PCR products were probed with non-radioactive Alkphos-labelled CaMV 35S promoter (1.3 kb *NotI/XhoI* restricted fragment of the plasmid pDK28) by Southern hybridization. Lane 1; 1 kb DNA step ladder. Lane 2: PCR control reaction containing water. Lane 3: PCR reaction containing DNA from the control wild-type (WT) plant. Lane 4: PCR reaction containing the plasmid pDK28 used as the positive control.

vector. The HotStart *Taq* DNA polymerase kit amplified the targeted two genes from the pDK28 transformation vector used as control. However, despite several optimizations of the PCR conditions, HotStart *Taq* DNA polymerase failed to amplify the genomic DNA of the peanut leaves. Successful amplification of the genomic DNA from the peanut leaves was achieved by using an alternative Rainbow DNA polymerase (ExtremoZyme, Huntsville, USA). As shown in Figure 3a, DNA bands corresponding to the expected size (1 kb) of the PCR products from the CaMV 35S promoter were amplified from peanut leaves DNA of kanamycin-resistant plants. Additional PCR bands were generated due to unspecific binding of the polymerase.

To confirm the authenticity of the bands, PCR products were transferred to nylon membrane for Southern hybridization. The DNA fragment of the CaMV 35S promoter used as

probe hybridized with the 1-kb band generated by PCR primers 1 and 2 (Figure 3b). Similar results were obtained with the *NPTII* probe which hybridized with the 0.8-kb band generated by primers 3 and 4 (Fig. not shown). No hybridization band was observed in PCR products obtained from leaves DNA of the control wild-type peanut plants. Hybridization bands were observed in 26 out of 59 (44%) kanamycin-resistant plants, implying that 44% of regenerated plants were putatively transgenic. There was no correlation between PCR positive plants and the number of pods produced. For example, plants #55 and #66 were both PCR positive, but they produced 32 and 3 pods, respectively, while plants #37 (not PCR positive) and #54 (PCR positive) produced 2 pods each.

Southern hybridization was performed on 10 of the selected plants to confirm their transgenic status and to determine the copy number of the *Ara h 2*-RNAi transgene. The plants were randomly selected from each group based on the number of pods they produced. The PCR negative plant #9 was also included. Genomic DNA was digested with *XhoI* which cuts within the T-DNA only once, and with a combination of *XhoI/NotI* which splices out the CaMV 35S promoter fragment from the T-DNA. Data presented in Figure 4. Corroborate with the PCR results. The CaMV 35S promoter fragment probe hybridized with the DNA from all selected putative transgenic plants, but not with the DNA from the PCR negative plant #9. Hybridization bands resulting from the double digest (D) lanes for plant #23, #45, #54 and #66 correspond to the expected size (1.3 kb) of the CaMV 35S promoter fragment, indicating the stable integration of the transgene in the peanut genome. Analyses of the single digest (S) reveal that all the selected transgenic plants [plant #12 (19 pods), plant #23 (9 pods), plant #32 (4 pods), plant #39 (11 pods), plant #45 (7 pods), plant #55 (32 pods), plant #66 (3 pods) and plant #6 (25 pods)] carried each a single inserts with a

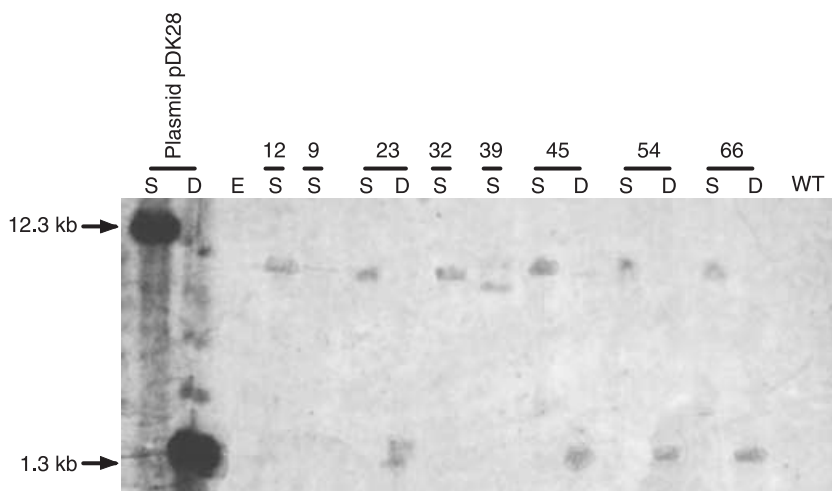


Figure 4 Southern blot analysis of selected putative transgenic peanut plants. The DNA was digested with *XhoI* to provide a single cut (S) within the T-DNA. A double cut (D) was also performed using *XhoI* and *NotI* to splice out the CaMV 35S promoter fragment and electrophoresed on 0.8% agarose. The blot was probed with non-radioactive Alkphos-labelled CaMV 35S promoter DNA fragment. Numbers represent the assigned number of the *Ara h 2*- T_0 plants. E, empty lane; WT, wild-type control DNA.

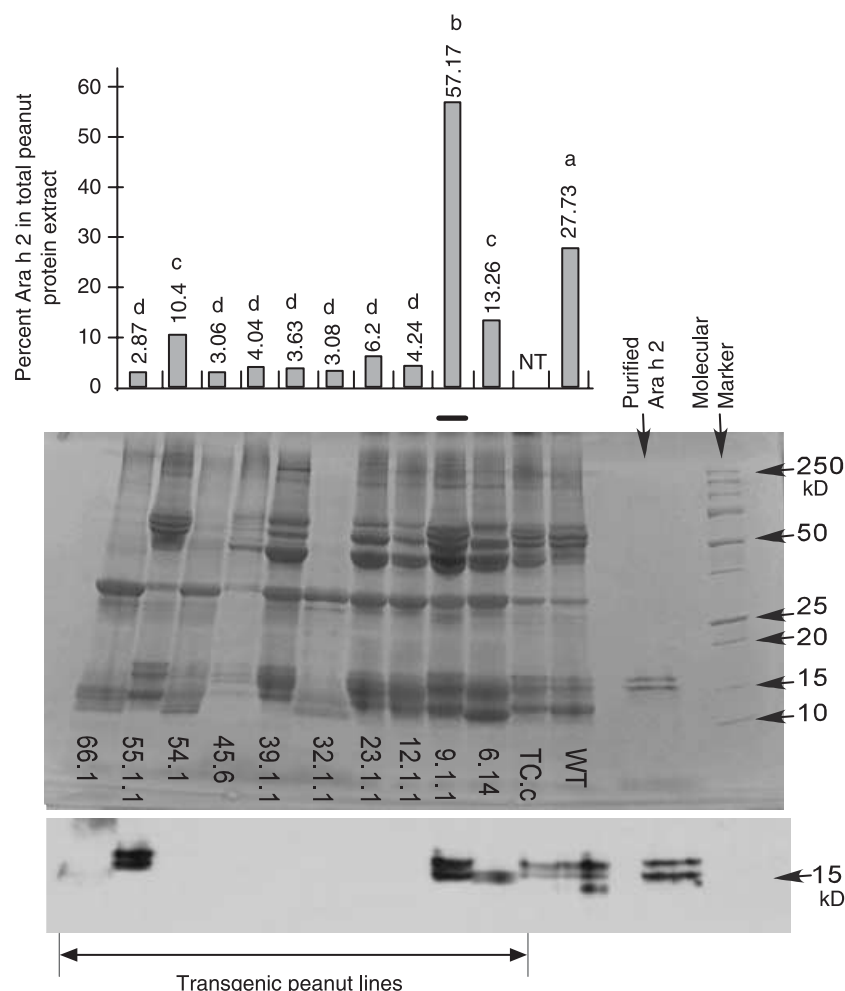


Figure 5 Determination of Ara h 2 in peanut samples. (a) Sandwich ELISA shows the percentage of Ara h 2 in peanut total protein extracts. Numbers followed by the same letters are not statistically different at $P < 0.05$. (b) SDS-PAGE shows the protein profiles and (c) Western immunoblot analysis in crude peanut extracts to detect the presence of the Ara h 2 protein. Hybridization was performed using an Ara h 2 specific monoclonal antibody. About 0.5 μ g purified Ara h 2 was loaded. WT, wild-type control sample; TCc, tissue culture non-transgenic control sample; (-), sample from a Southern negative plant; NT, not tested.

similar pattern of gene integration. Only plant #39 showed a slightly different pattern of integration (Figure 4).

Sandwich ELISA using Ara h 2-mAbs revealed a significant reduction of Ara h 2 content of crude peanut extracts

Seventy-one seeds were randomly selected from pods collected from the transgenic plants #6, #12, #23, #32, #45, #54, #55 and #66. Crude peanut extract (CPE) from each of the selected seeds was analysed by sandwich ELISA to determine the Ara h 2 content in the total peanut protein extracts. The percentage of allergen Ara h 2 in the total protein extracts was different amongst the samples. There was a significant difference ($P < 0.05$) in the percentage of Ara h 2 content measured for the wild-type control which contained 27.73% and the selected transgenic seeds which contained from 2.87% up to 6.24% Ara h 2 (Figure 5a). Compared to the wild type, there was a reduction of Ara h

2 content of at least 21.69% up to 25.06%. Sample 9.1.1. from the Southern negative plant #9 was the highest in Ara h 2 content with 57.17%.

Western immunoblotting revealed the absence of Ara h 2 in several transgenic peanut seeds

To further confirm the ELISA data, SDS-PAGE and Western immunoblot analyses were performed. The characteristic Ara h 2 doublet migrated on SDS-PAGE as expected around 17 kDa in the purified Ara h 2 protein sample. This doublet was also visible in the protein profiles in CPE of the two controls wild type and tissue culture, as well as in the CPE of seeds from some transgenic and non-transgenic plants. It is interesting to note that the protein profile in SDS-PAGE of some transgenic samples were different from that of the wild-type controls (Figure 5b).

Hybridization with the Ara h 2 mAb detected the 0.5 μ g of the 17 kDa purified Ara h 2 protein sample, and also

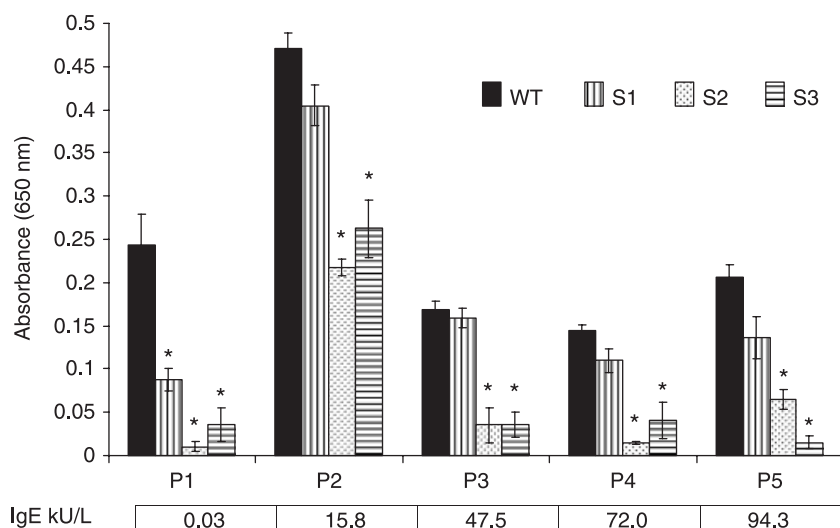


Figure 6 Results of indirect immunoassay with extracts of wild type (WT) and transgenic peanut seeds 12.1.1 (S1), 32.1.1 (S2) and 45.6 (S3). * indicates the samples is significantly lower than the control samples ($P < 0.05$). P, selected patients sera containing different concentration of IgE in kU/L.

confirmed the presence of the immunodominant Ara h 2 protein in the controls wild type and tissue culture (Figure 5c). There is a correlation between Southern, ELISA, SDS-PAGE and Western blot data. On the Western blot, Ara h 2 was not detected in CPE of all the transgenic seeds containing 2.87 up to 6.2% Ara h 2 in the total protein extract, while positive signals for Ara h 2 were shown for lines 6.14 and 55.1 which had the highest percentage of Ara h 2 content among the transgenic lines. A total of 16 of the 71 seeds screened (22.5%) from transgenic plants #6 (2/15), 12 (4/10), 23 (1/5), 32 (1/5), 39 (1/5), 45 (3/9), 52 (0/8), 54 (1/3), 55 (2/6) and 66 (1/5) showed no detection of Ara h 2 or significantly faint band intensity (not shown). Ara h 2 was detected in all 5 CPE samples of the Southern negative plant #9.

Indirect ELISA using patient's sera shows a decrease in IgE binding capacity of transgenic peanut seeds

The IgE binding capacity of transgenic peanut seeds was compared to that of the wild type using sera from five patients with different levels of severity of documented peanut allergy. Data in Figure 6 show that overall the IgE binding capacity of CPE of all transgenic peanut samples is lower than wild-type control. More specifically, IgE binding capacity of transgenic samples S2 (32.1.1) and S3 (45.6) is strongly reduced and significantly lower ($P < 0.05$) than that of the wild type for all five patients regardless of the serum IgE titre. For the equivocal patient P1 (serum IgE = 0.03 kU/L) this significant reduction is observed with all three selected transgenic seeds (12.1.1; 32.1.1; 45.6). For patients P2 (IgE = 15.8), P3 (IgE = 47.5), P4 (IgE = 72.0) and P5 (IgE = 94.3) the IgE binding capacity was significantly reduced for both

transgenic peanut samples S2 and S3. These data corroborate with previous results obtained with Sandwich ELISA performed using Ara h 2-mAb (Figure 5a) and the Western blot analysis (Figure 5c), and also suggest a significant decrease in the allergenicity or allergenic potency of the selected transgenic peanut seeds.

Discussion

Peanut allergy is the most deadly cause of food-induced anaphylaxis (Bock *et al.*, 2001) and remains a serious challenge to the peanut and food industries. In this study, we have demonstrated a significant reduction in the level of Ara h 2, the most immunodominant peanut allergen (Koppelman *et al.*, 2001; Knol *et al.*, 2003) in the first-generation transgenic (T_0) peanut using RNAi. This reduction of the Ara h 2 level has resulted in a significant decrease in the allergenicity or allergenic potency (expressed as IgE binding capacity) of selected transgenic seeds using sera from individuals allergic to peanut.

The *Agrobacterium*-mediated transformation protocol described by Egnin *et al.* (1998) was successfully applied in this study to produce fertile transgenic peanuts within 7 months. This success also confirmed that the lack of fertility of the transgenic plants we previously produced (Konan *et al.*, 2004) using microprojectile-mediated transformation of somatic embryos was mostly due to the lengthy (> 10–15 months) tissue culture involved in that protocol. However, the *Agrobacterium*-mediated transformation procedure used requires further improvement as it produces high rate of escapes (over 50%) of regenerated plants due to the delayed selection.

The presence and integration of the Ara h 2 transgene in the peanut genome was tested by PCR and Southern hybridization of T₀ kanamycin resistant plants. PCR analysis amplified a 1-kb fragment from the heterologous CaMV 35S promoter in 44% (26/59) of kanamycin-resistant plants. It is noteworthy that the newly identified polymerase 'Rainbow' (ExtremoZyme, Huntsville, AL, USA) was more efficient at amplifying the targeted 1-kb CaMV 35S promoter fragment in peanut genomic DNA as compared to the standard *Taq* polymerase (Invitrogen, Carlsbad, CA, USA). Southern analysis of the CaMV 35S promoter confirmed the insertion of the transgene in the genome of the selected 10 putative transformants. One copy transgene was identified in all transformants which presented similar integration pattern except for plant #39, which suggests that it resulted from an independent transformation event. *A. tumefaciens*-mediated plant transformation is notoriously known for its low copy transgene integration (Hiei *et al.*, 1994; Mohanty *et al.*, 1999).

In this study, we have also demonstrated for the first time the successful application of RNAi to peanut, targeting the elimination of the immunodominant allergen Ara h 2 from seeds. RNAi is the most successful method currently available to reduce or eliminate a gene product or protein for plant quality improvement. It triggers gene-specific silencing based on sequence homology-dependent degradation of cognate mRNA (Matzke *et al.*, 2001; Hannon, 2002). The technology has been successfully used to silence allergen genes in rice (Tada *et al.*, 1996), soybean (Herman *et al.*, 2003), apple (Gilissen *et al.*, 2005) and tomatoes (Lorenz *et al.*, 2006). For the first time, it has been applied to peanut, the most severe and most deadly cause of food allergy with the aim to render this crop safer and more nutritionally fit.

The insertion of the transgene did not adversely affect the agronomic performance of the transgenic peanuts as compared to the controls. No phenotypic differences were observed between transgenic plants and non-transgenic culture-derived tissue culture and wild-type controls for plant morphology, growth rate and reproduction. All plants flowered at the same time and produced pods which contain either one or two seeds each. Although a wide variation was observed in the number of pods produced per plants, there was, however, no correlation between the number of pods and the transgenic status of the plants.

The efficiency of eliminating Ara h 2 from transgenic peanuts was monitored by ELISA and Western immunoblot analyses. Sandwich ELISA performed using Ara h 2-mAbs, corroborated with results obtained with the Western immunoblots, and clearly demonstrated the effectiveness of the

pHANNIBAI-based RNAi transformation vector for gene silencing in plants (Wesley *et al.*, 2001). The data reported here were obtained from 71 seeds pooled from 43 randomly selected pods of 10 transgenic plants. When combining the results obtained with the ELISA and Western immunoblotting, we can confirm that 16 out of the 71 (22.5%) total transgenic seeds screened are putatively free of Ara h 2 and/or have a significant reduction in their Ara h 2 content. Additional screening would probably identify more Ara h 2-reduced transgenic seeds.

A key observation was the difference in the pattern of silencing for two seeds from the same pod. There were instances of a significant reduction of Ara h 2 in only one of the two seeds. We attributed these differences to the heterozygosity of the transgene in this first generation T₀ transformants. Previous studies have shown that the percentage and degree of gene silencing increased in the progeny as the plants become homozygous for the transgene (Tada *et al.*, 1996; Aida *et al.*, 1998) and that homozygosity enhances the silent phenotype. This was also recently demonstrated in tomato with the elimination of the *Lyc e 3* allergenic protein (Lorenz *et al.*, 2006). Therefore, we are expecting an increase in the number of pods with the two seeds free of Ara h 2 in the T₁ and T₂ progenies of the transgenic T₀ peanut plants.

The allergenic potency of the transgenic peanut expressed as IgE binding capacity was evaluated with ELISA performed using serum of patients with a history of peanut allergy at different degree of severity. We selected patients with different levels of IgE in their serum to determine individual response to the selected transgenic peanut seeds compared to the control wild type. The results showed a significant reduction of the immunodominant Ara h 2 allergen protein in transgenic plants which translated in a significant decrease in allergenicity or allergenic potency of transgenic seeds. All five patients displayed similar trends in their responses with significantly lower IgE binding capacity for transgenic seeds 32.1.1 and 45.6. Together, Southern, ELISA and Western results demonstrated a putative elimination and/or a significant reduction in the content of the immunodominant Ara h 2 allergen protein, resulting in a significant decrease in allergenicity of first generation transgenic seeds.

Ara h 2-T₀ seeds have been planted and further molecular and immunological analyses will be performed in the next generations of seeds using *in vivo* and *in vitro* assays. The nutritional and sensory properties of the seeds will also be determined. Fostering the utilization of hypoallergenic peanut especially with the food and confectionery industry could reduce severe allergic reactions and fatalities in the event of an accidental ingestion.

Experimental procedures

Construction of the peanut Ara h 2 silencing vector pDK28 and mobilization into *A. tumefaciens* EHA105

Two PCR fragments of 265 bp each were generated from the Ara h 2 genomic DNA, and cloned as *XhoI/KpnI* and *XbaI/HindIII* fragments in the cloning sites of pHANNIBAL (Wesley et al., 2001) to create an inverted repeat transgene separated by the pdk intron. The inverted repeat Ara h 2 recombinant DNA was linked to the cauliflower mosaic virus CaMV 35S promoter and to the Octopine Synthase (OCS) terminator in pHANNIBAL. The transformation cassette was spliced out of pHANNIBAL by a *NotI* restriction digest and subcloned into the unique *NotI* site of the binary plasmid pART27 (Gleave, 1992) containing the *NPTII* selection marker gene. The resulting plasmid pDK28 was mobilized into *A. tumefaciens* EHA 105 as described by Egnin et al. (1998).

Plant Materials, transformation and production of fertile Ara h 2- T_0 plants

Preparation of hypocotyls explants

Peanut variety 'Georgia green', a Runner type was purchased from Growth South (Montgomery, USA). Peanut seeds were sterilized in 20% (v/v) commercial Chlorox and germinated on MSTDZ medium (Egnin et al., 1998). Culture media were adjusted to pH 5.8, and 3 g/L phytigel (Sigma Chemical, St. Louis, MO, USA) was added before autoclaving. Cultures were incubated in a Percival growth chamber (Percival Scientific, Boone, IA, USA) at 27 °C under fluorescent lighting in a 16/8 h (light/dark) photoperiod.

Transformation and regeneration of fertile kanamycin-resistant Ara h 2- T_0 plantlets

Hypocotyl explants of about 0.5 cm long were excised from 6-day germinated peanut embryos. Explants were infected by immersion in a suspension of *A. tumefaciens* strain EHA105 harbouring the transformation vector pDK28. Explants were removed from the bacterial solution, blotted on sterile towel papers and cocultured on hormone-free MSO medium [MS medium + 100 mg/L myo-inositol + 30 g/L sucrose] for 5 days.

Following the cocultivation period, infected explants were transferred on MSTDZ regeneration medium supplemented with 400 mg/L carbenicillin until shoot regeneration. On the other hand, cocultivated explants were transferred on the selection medium [MSO medium + 200 mg/L carbenicillin + 200 mg/L kanamycin] immediately following coculture, or one to 3 weeks after the coculture. Shoots of about 0.5–1 cm height were excised and transferred to 100 × 25 mm Petri dishes containing the selection medium. Each shoot was assigned an identification number. After 4–6 weeks, shoots resistant to kanamycin were transferred to Magenta boxes (77 × 77 × 97 mm) containing the rooting medium [MSO medium + 50 mg/L kanamycin and 100 mg/L carbenicillin].

Transfer of peanut Ara h 2- T_0 plantlets to soil

Rooted plantlets were hardened for 1 week in Percival growth chamber. The lids of the Magenta culture vessels were removed and 10 mL of sterile distilled water was added to the surface of the solid rooting

medium. The putative transgenic plants were transferred into 6" greenhouse plastic pots containing 50% Metro Mix (Hummert International, St. Louis, MO, USA) and 50% sand. The plants were incubated in an Enconair growth chamber model A60 (Enconair, Winnipeg, MB, Canada) set at 28 °C for 16 h in light, and 24 °C for 8 h in dark and 85% ventilation during the first 3–5 weeks for acclimation of the young plants. The plants were watered twice a week and Peters 20-10-20 fertilizer at 5 g per gallon was added to the watering solution once a month.

PCR analysis of kanamycin-resistant plants

Fresh young leaves from kanamycin-resistant peanuts were collected from the growth chamber and immediately stored at –80 °C until used. DNA extraction was performed with about 1 g of leave tissue using the Qiagen plant DNA maxiprep kit (Qiagen, Valencia, CA, USA). PCR reactions were performed to target both the CaMV 35S promoter and the *NPTII* selection marker gene not naturally present in peanut. Primer 1 5'-CAGAGGCAAGAGCAGCAGC-3' and primer 2 5'-GCTGGGGTATCGATCACTGTCAATGG-3' were designed to specifically amplify a 1-kb DNA fragment from the CaMV 35S promoter, while primers 3 5'-CCTATTCCGCCCGGATCCG-3' and primer 4 5'-GTCAAGAAGGCGATAGAAGGCG-3' were designed to amplify a 0.8 kb DNA fragment of the *NPTII*. PCRs were performed in a total volume of 25 µL using the HotStar Taq DNA polymerase kit (Qiagen) and a MJ Research MiniCycler. After the initial 15 min at 95 °C required to start the Taq polymerase, 30 amplification cycles were performed as follows; denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, primer extension at 72 °C for 2 min, and final elongation step at 72 °C for 10 min. When the Rainbow DNA polymerase (ExtremoZyme) was used instead of Taq polymerase, the reaction mixture consisted of 2.5 µL 10× buffer (10 mM Tris-HCl pH 8, 60 mM, KCl, 2 mM MgCl, 0.1% Triton X-100), 0.25 µL of 2 mM dNTP mix and 0.2 unit of the polymerase. PCR products were separated on 0.8% (w/v) agarose gels. To verify amplification of the targeted genes, the fragments separated on the agarose gels were transferred on to Immobilon-Ny+ membrane (Amersham Biosciences, Piscataway, NJ, USA) and probed with CaMV 35S promoter and *NPT II* gene fragments obtained from the plasmid pDK28 (Figure 1). DNA was transferred on to the membrane by the capillary transfer method (Southern and Maskos, 1994). Prehybridization (30 min) and hybridization (36 h) were performed at 55 °C in a hybridization oven (Biometra OV3, Goettingen, Germany), followed by 2 washes at 65 °C. For probe preparation, the 1.3 kb CaMV 35S promoter was spliced out of plasmid pDK28 with *NotI/XhoI* digestion and the *NPTII* DNA fragment was a PCR product amplified from the plasmid pART27 using primers 3 and 4. The CaMV 35S promoter fragment and the *NPTII* PCR products were gel purified, extracted in phenol/chloroform and used for labelling. Labeling of the probes and the signal detections were performed using the non-radioactive Alkphos direct labelling and detection kit (Amersham Biosciences). The membrane was exposed to Hyperfilm ECL (Amersham Biosciences) for 2–4 h at room temperature. The X-ray film was developed with Agfa CP-1000 Table Top film processor.

Southern blot analysis

The genomic DNA (3–5 µg) from 10 putative transformants and from one PCR negative plant (plant #9) was digested to identify the

integration and copy number of the transgene in the peanut genome. Single digest was performed with *XhoI* which has one unique restriction site within plasmid pDK28 (Figure 1). Double digests were performed with a combination of *XhoI* and *NotI*. Digested DNA was electrophoresed at 70 V for 4 h on a 0.8% agarose gel. Separated DNA fragments were transferred on to Immobilon-Ny⁺ membrane (Amersham Biosciences) by capillary transfer method. The 1.3 kb CaMV 35S promoter was labelled with non-radioactive AlkPhos direct system and used as probe. Labeling, hybridization and detection were performed as described previously for hybridization and detection of the PCR products.

Immunological analyses

Total peanut protein extraction

Peanut pods were collected from the putative transgenic and control plants as they matured and kept at -20°C until used. Two to five pods were randomly selected from each plant, and seeds from the selected pods were used for protein extraction. Total protein was extracted from each seed as described by Koppelman *et al.* (2001). CPE from each plant were numbered based on the assigned number of the plant and the assigned number of the pod, supplemented with 1 or 2 to account for seed #1 and #2, respectively, in each pod. For pods containing only one seed, the CPE were numbered using only the assigned number of the plant, and the assigned number of the pod. Total protein concentration was determined using the Coomassie Plus (Bradford) Assay kit (Pierce Biotechnology, Rockford, IL USA), and the CPE were stored at -20°C until used.

Quantification of Ara h 2 using sandwich ELISA

Quantification of Ara h 2 in peanut samples was performed using an Ara h 2 ELISA kit as described by the manufacturer (Indoor Biotechnologies, Charlottesville, VA USA). Polystyrene 96 wells plates (Fisher Scientific, Suwanee, GA, USA; cat. no. 12565135) were coated with Ara h 2 mAb 1C4 diluted at 1 : 1000 in 50 mM carbonate-bicarbonate buffer pH 9.6 and incubated overnight at 4°C . In the following steps, reactions were carried out at room temperature. Between each step the wells were washed 3x with 100 μL PBS-0.05% Tween 20, pH 7.4 (PBS-T) by pipetting up and down the wash Buffer. The wells were blocked for 30 min by adding 100 μL 1% BSA PBS-T per well. After washing, Ara h 2 protein standard (ST-AH2 at 2500 ng/mL) and diluted CPE were added to the wells and incubated for 1 h. Ten serial dilutions of the Ara h 2 protein standard were performed in 1% BSA PBS-T according to the manufacturer's instruction to establish a standard curve. CPE were adjusted to 500 $\mu\text{g/mL}$ total proteins, and diluted to 1 : 10 000 in 1% BSA PBS-T before adding at 100 $\mu\text{L/well}$. After washing, biotinylated pAb rabbit anti-Ara h 2 (PA-AH2) was diluted 1 : 1000 in 1% BSA PBS-T and added at 100 $\mu\text{L/well}$ and incubated for an additional 1 h. For detection, HRP-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA; cat no. 111-036-045) was diluted at 1 : 4000 and added at 100 $\mu\text{L/well}$ for 30 min incubation. The wells were then washed 6x with PBS-T to remove residual traces of HRP. The assays were developed by adding 100 $\mu\text{L/well}$ 1 mM 2,2'-azino-di (3 ethylbenzthiazoline sulphonate) (ABTS, Sigma, St. Louis, MO, USA; cat. no. A1888) in 70 mM citrate buffer, pH 4.2 containing 1 : 1.000 dilution of 30% H_2O_2 (Fisher Scientific, cat. no. H325-100). The plates were read when the optical density at 405 nm reached 2

for the Ara h 2 protein standard. Each sample was prepared in triplicate and the mean values were compared using one-way ANOVA (SPSS 12.0 for Window, SPSS Inc., Chicago, IL, USA). Differences were calculated at a probability level of significance of $P < 0.05$ using Tukey HSD.

SDS-PAGE and Western immunoblotting using Ara h 2 mAb

Aliquots of 75 μL of the CPE were mixed with 25 μL of 4x sample buffer, and heated in a water bath at 65°C for 15 min. Fifteen micrograms of the denatured protein samples was loaded in the wells of 10–20% precast gel. Five microlitre of the molecular weight marker 'Precision plus Protein' was loaded per gel. Purified Ara h 2 protein (0.5 μg) was loaded and used as reference. Each sample was loaded on duplicate gel and proteins were separated by SDS-PAGE at 200 V for 1 h using a Criterion Cell electrophoresis tank. One gel was stained with Coomassie Brilliant Blue R-250, and the second unstained gel was blotted for 2 h on to a PVDF membrane sheet using a Bio-Rad Trans-Blot.

The blots were blocked for 1 h with 5% dry milk in TBS containing 0.05% Tween 20 and probed 50 min with Ara h 2 mAb used at 1 : 6000 dilution. The blots were developed using the Immuno star goat anti mouse-horseradish conjugated (GAM-HRP) detection kit. Solutions and ingredients were purchased from Bio-Rad (Hercules, CA, USA) and protocols used followed the manufacturer's instructions.

Estimation of IgE binding capacity of transgenic peanuts using Indirect ELISA and sera of peanut allergic patients

Human sera of patients allergic to peanut, with known IgE (kU/L) levels were classified as described in Table 1 following the ImmunoCAP quantification scoring guide (Pharmacia, London, UK). Patients were selected from class 0/1 (equivocal; IgE = 0.1–0.34 kU/L), class 3 (high positive; IgE = 3.5–17.4 kU/L), class 4 (very high positive IgE = 17.5–49.9), and class 5 (very high positive, IgE = 50–99.9 kU/L).

Crude protein extract were diluted 1 : 200 in carbonate buffer (pH 9.6) and added to Costar polystyrene 96-well EIA plates (Corning Inc., Corning, NY, USA) at 50 $\mu\text{L/well}$. The plates were incubated at 37°C for 1 h. After washing the plates with PBS-T, 200 μL of 1% bovine serum albumin (BSA) in PBS were added to each of the wells. After incubation at 37°C for 1 h, the plates were washed and 50 μL of human serum (1 : 20 diluted in 1% BSA/PBS-T) were added to the wells and incubated for 1 h at 37°C . After washing the plates three times with PBS-T, 50 μL of mouse antihuman IgE peroxidase

Table 1 Quantitative scoring guide of allergen-specific IgE testing ImmunoCAP (Pharmacia, London, UK) and selected patient sera.

Class	IgE (kU/L)	Comment	Selected patient sera
0	< 0.10	Negative	
0/1	0.1–0.34	Equivocal	P1 IgE = 0.03 kU/L
1	0.35–0.69	Low positive	
2	0.70–3.4	Moderate positive	
3	3.5–17.4	High positive	P2 IgE = 15.8 kU/L
4	17.5–49.9	Very high positive	P3 IgE = 47.5 kU/L
5	50.0–99.9	Very high positive	P4 IgE = 72.0 kU/L P5 IgE = 94.3 kU/L
6	> 100		

conjugate (Zymed, South San Francisco, CA, USA), 1 : 40 000 diluted in 1% BSA PBS-T were added to each of the wells. Plates were incubated for 1 h at 37 °C and then washed five times with PBS-T. The liquid enzyme substrate -3, 3', 5, 5'-tetramethyl-benzidine (Sigma) was added to the plates at 100 µL/well and the plates were incubated at room temperature for 30 min. Colour development was measured at 650 nm using a Benchmark Plus Microplate Spectrophotometer (Bio-Rad). The differences of IgE binding between transgenic and control CPE on the same patient's serum were compared by paired-samples *t*-test using SPSS 12.0 for Windows (SPSS Inc.).

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