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Reduction of IgE Binding and Nonpromotion of *Aspergillus flavus* Fungal Growth by Simultaneously Silencing Ara h 2 and Ara h 6 in Peanut

YE CHU,[†] PAOLA FAUSTINELLI,[†,#] MARIA LAURA RAMOS,[†,⊥] MARTIN HAJDUCH,[‡,||] SEVERIN STEVENSON,[‡] JAY J. THELEN,[‡] SOHEILA J. MALEKI,[§] HSIAOPO CHENG,[§] AND PEGGY OZIAS-AKINS*,[†]

Department of Horticulture, University of Georgia Tifton Campus, Tifton, Georgia 31793; Department of Biochemistry, University of Missouri—Columbia, Columbia, Missouri 65211; and USDA-ARS-Southern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, New Orleans, Louisiana 70124

The most potent peanut allergens, Ara h 2 and Ara h 6, were silenced in transgenic plants by RNA interference. Three independent transgenic lines were recovered after microprojectile bombardment, of which two contained single, integrated copies of the transgene. The third line contained multiple copies of the transgene. Ara h 2 expression was significantly suppressed in all three lines, whereas Ara h 6 was reduced in two lines. Expression of peanut allergens Ara h 1 and Ara h 3 was not noticeably affected. Significant reduction of human IgE binding to Ara h 2 and Ara h 6 also was observed. Seed weight and germination data from transgenic and nontransgenic segregants showed no significant differences. Data collected from in vitro *Aspergillus flavus* infection indicate no significant difference in fungal growth between the transgenic lines and the nontransgenic controls. These data suggest that silencing Ara h 2 and Ara h 6 is a feasible approach to produce hypoallergenic peanut.

KEYWORDS: Ara h 2; Ara h 6; peanut allergen; gene silencing; *Aspergillus flavus*

INTRODUCTION

Peanut is an inexpensive and nutritious food source, rich in protein, carbohydrates, minerals, and other nutrients. However, consumption of peanut causes IgE-mediated allergic reaction in 0.6% of the total population and in 0.4−0.8% of children in the United States (*1*). A five-year study of 2878 children in England showed that reported peanut allergy is rising and more children are being sensitized to peanut (*2*). More than 80% of anaphylactic reactions are caused by peanut and other tree nuts (*3*). As little as 3 mg of peanut protein can induce allergic reaction in 50% of allergic patients (*4*). In most cases, peanut allergy persists throughout life (*5, 6*).

Eleven peanut proteins, Ara h 1−11, have been identified as allergens (*7, 8*). Ara h 2 and Ara h 6 are conglutins in the prolamin family that were suggested to be the most potent peanut allergens (*9*). A high percentage (80−90%) of peanut allergic patient sera has Ara h 2 and Ara h 6 specific IgE antibodies (*9, 10*), and the recognition of these allergens persists for more than a year after food challenge in children (*9*). Both Ara h 2 and Ara h 6 are heat stable and resistant to digestive enzymes such as trypsin, chymotrypsin, and pepsin (*11, 12*). The two proteins share 63% sequence homology (*13*), and IgE binding cross-reactivity between them has been reported (*14*). Expression of Ara h 2 and Ara h 6 is seed specific and regulated during seed development (*13, 15, 16*). Two genes for Ara h 2 have been identified, with Ara h 2.02 having an extra 12 amino acids compared to Ara h 2.01 (*17*). Ara h 2.01 and Ara h 2.02 are encoded by homeologous genes belonging to the two subgenomes of cultivated, tetraploid peanut and have molecular sizes of 18.8 and 20.2 kDa, respectively. Similarly, multiple (three) genes for Ara h 6 are present in tetraploid peanut (*13*). Functionally, Ara h 2 was shown to have weak trypsin inhibitor activity and partially protected Ara h 1 from digestion by trypsin (*18*). Protease inhibitors have been suggested to provide protection to plants under stress conditions such as fungal invasion (*19*), although there is little direct evidence for an in planta role of protease inhibitors in pathogen resistance (*20*). Aflatoxin, a potent human carcinogen, is produced by *Aspergillus flavus*, a fungus that infects major crops such as corn, cotton, and peanut. Expression of a 14 kDa trypsin inhibitor in maize was shown to be associated with host resistance to aflatoxin

* Corresponding author [telephone (229) 386-3902; fax (229) 386-7371; e-mail pozias@uga.edu].
[†] University of Georgia Tifton Campus.
[#] Present address: Catholic University of Cordoba, Camino a Alta Gracia Km 7A 1/2 (5017) Cordoba, Argentina.
[⊥] Present address: Department of Plant, Soil and Agricultural Systems, Southern Illinois University, Carbondale, IL 62901-4415.
[‡] University of Missouri—Columbia.
[||] Present address: Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, Nitra, Slovak Republic.
[§] U.S. Department of Agriculture.

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production (*21*). Silencing of this 14 kDa trypsin inhibitor in maize promoted *A. flavus* growth and aflatoxin production (*22*).

Multiple approaches have been developed to mitigate peanut allergy such as modified peanut allergen peptide immunotherapy, plasmid DNA immunotherapy, and IgE inhibition (*3, 23*). Some of them show promise in in vitro or animal models (*24, 25*); however, none of them has been applied clinically due to the high risk of anaphylactic response from patients. We chose to address the peanut allergy issue by knocking down expression of the major peanut allergens through RNA-mediated posttranscriptional gene silencing (*26*). Previously, this approach was applied to reduce allergens in several plants such as a soybean allergen P34 (*27*), a 14−16 kDa rice allergen (*28*), an apple allergen Mal d 1 (*29*), tomato allergens Lyc e 1 and Lyc e 3 (*30, 31*), and a ryegrass allergen Lol p 5 (*32*). The effect of Ara h 2 knockdown on susceptibility of peanut to *A. flavus* also was evaluated in this study.

MATERIALS AND METHODS

Plasmid Construct. The *ara h 2* RNAi plasmid illustrated in **Figure 1A** was constructed from pFGC1008 (AY310333) provided by the *Arabidopsis* Biological Resource Center, Ohio State University. It contains a CaMV 35S promoter cassette with the OCS terminator and a hygromycin resistance cassette with the mannopine synthase 1′ promoter. A 335 bp GUS fragment serves as the linker for the inverted repeat to facilitate hairpin RNA formation. A 242 bp fragment of the GFP gene was cloned upstream of the sense *ara h 2* repeat for use in another study. The 293 bp sense *ara h 2* fragment of the inverted repeat was 100% identical to nucleotides 122−414 of L77197 (*ara h 2.01*). The 228 bp antisense *ara h 2* fragment of the inverted repeat was 100% identical to nucleotides 192−419 of L77197 (**Figure 1B**). The two *ara h 2* fragments can form a 222 bp inverted repeat structure upon transcription. This portion of *ara h 2.01* spans the insertion in *ara h 2.02*, and there are two SNPs between the two genes in the flanking regions.

Plant Tissue Culture. Embryogenic cultures of peanut were initiated from mature zygotic embryos of 'Georgia Green'. Seeds were surface sterilized in 70% ethanol for 3 min followed by immersion in 40% Clorox on a shaker for 20 min. After three rinses with sterile deionized water, the plumule region of the embryo axis was excised under a stereomicroscope and cultured on embryo induction medium, which consisted of FN lite macro salts (*33*), MS (*34*) micronutrients, B5 vitamins (*35*), 1 g/L L-asparagine (Fisher Scientific, Pittsburgh, PA), 3 mg/L picloram (Dow Agrosciences, Indianapolis, IN), 3% sucrose (J. T. Baker Inc., Phillipsburg, NJ), and 0.8% agar (Sigma, St. Louis, MO). The pH was adjusted to 5.8 prior to autoclaving at 121 °C for 20 min. After the medium was cooled to 65 °C, it was supplemented with filter-sterilized L-glutamine (1 g/L) (Acros Organics, Morris Plains, NJ). Embryogenic cultures were grown in the dark at 26 ± 2 °C for up to 9 months and bombarded within 2 weeks after subculture.

Microprojectile Bombardment, Selection, and Regeneration. Plasmid DNA was isolated using the Qiagen Plasmid Maxi/Midi Kit (Qiagen, Valencia, CA) and quantified with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA). Microprojectile bombardment was carried out as described previously (*36*) except that 0.6 μm gold particles (Bio-Rad, Hercules, CA) were used. Hygromycin-resistant embryogenic tissues were regenerated on MS medium supplemented with 1 mg/L thidiazuron (NOR-AM Chemical Co., Wilmington, DE), 20 mg/L hygromycin (Sigma), 3% sucrose, and 0.8% agar under a daily photoperiod of 16 h of light (50 μmol/m^2/s) and 8 h of darkness. Germinating embryos were transferred to MS medium containing 20 mg/L hygromycin for an additional 4 weeks. Embryos with well-developed shoot−root axes were transferred to ventilated culture vessels containing MS medium plus 0.5 mg/L kinetin (Sigma) and 0.25 mg/L 6-benzylaminopurine (Sigma). Elongated shoots were rooted in Magenta vessels on MS medium containing 0.1 mg/L 1-naphthaleneacetic acid (Sigma). Rooted plantlets were transplanted to potting mix and acclimatized to greenhouse conditions.

PCR Analysis. DNA was extracted from leaf tissue as described previously (*37*). Selection of transgenic plants was carried out by amplifying a 790 bp fragment of the inverted-repeat cassette with GFP sense primer 5′-CAA GGA CGA CGG CAA CTA CAA GA-3′ and GUS antisense primer 5′-AGA ACA TTA CAT TGA CGC AGG TGA-3′. Amplification was carried out by denaturing at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 59.3 °C for 30 s, and 72 °C for 60 s with an extension of 7 min at 72 °C and a 4 °C soak.

Southern Blot Analyses. Genomic DNA (20 μg), extracted according to a modified CTAB protocol (*38*), was digested with *Hin*dIII, which cut the plasmid once at the 3′ end of the OCS terminator. After electrophoresis on 0.8% agarose gel, DNA was transferred to GeneScreen Plus nylon membrane (NEN Research Products, Boston, MA). A 291 bp GUS linker probe and an 870 bp *ara h 2* probe were labeled with [^{32}P]-dCTP and used for hybridization. The Southern blot image was captured with a Storm phosphor imager (Amersham Biosciences, Pittsburgh, PA).

Western Blot Analyses. Seed protein was extracted (*39*) and quantified according to the Bradford protein assay (Bio-Rad). Protein (10 μg) from each sample was separated by electrophoresis on a 5% polyacrylamide gel or a 4−20% Novex Tris-HCl gradient gel (Invitrogen). Protein was subsequently electroblotted onto a PVDF membrane and reacted with chicken anti-Ara h 1 (1:5000 v/v), Ara h 2 (1:8000), Ara h 3 (1:5000), or Ara h 6 (1:5000) antibodies, custom manufactured by Sigma Immunosys (Woodlands, TX) and provided at a concentration of 1 μg/mL. Subsequently, goat anti-chicken secondary antibody (1:100,000) conjugated with horseradish peroxidase (Sigma) was applied, and signal detection was performed with the ECL-Plus kit (GE Healthcare, Piscataway, NJ). For IgE Western blots, membranes were incubated overnight with a 1:10 dilution of patient sera in PBS-T. Sera were from documented peanut allergic individuals with either a positive food challenge or a convincing history of peanut allergy. Sera from allergic individuals were collected at the University of Arkansas for Medical Sciences, Little Rock, AR, approved and in accordance with the rules and regulations of the institutional review board. Goat anti-human−IgE horseradish peroxidase (HRP)-labeled secondary antibody was applied at 1:100,000 or 1:10,000. Signal was detected with an ECL-Plus kit (GE Healthcare).

Quantitative Immunoblotting. Total protein from one cotyledon was isolated as described previously (*40*), and the pellet was resuspended in SDS-PAGE sample buffer [0.06 M Tris-HCl (pH 6.8), 0.06 M sodium dodecyl sulfate, 10 mM dithiothreitol, 5% glycerol (v/v), and 0.0012 mM bromophenol blue]. Resuspended protein was quantified using a BCA assay kit (Pierce, Rockford, IL) per the manufacturer's instructions. Proteins (20 μg) were separated using 12% polyacrylamide gels in the Hoefer miniVE system (GE Healthcare). Proteins were transferred to Hybond, low fluorescing PVDF (GE Healthcare) in transfer buffer [25 mM Tris-Base, 192 mM glycine, 10% (v/v) methanol] at 200 mA for 6 h.

Blots were rinsed in PBS-T [10 mM NaH_2PO_4, 150 mM NaCl, 0.3% (w/v) Tween 20, pH 7.2] for 10 min, blocked for 1 h in PBS-TM [PBS-T plus 3% (w/v) nonfat dried milk], and then probed with primary antibody (Ara h 2 and Ara h 6 raised in chicken) at 1:8000 dilution in PBS-TM for 16 h, all performed at 4 °C. Blots were washed in PBS-TM twice for 5 min, rinsed in PBS-T twice for 5 min, blocked in PBS-TM for 1 h, and then incubated with secondary antibody (goat anti-chicken IgY-HRP, Santa Cruz Biotechnology, Santa Cruz, CA) in PBS-TM at 1:20,000 dilution for 2 h at 25 °C. Blots were washed three times for 5 min each in PBS-TM, and once in PBS-T for 5 min before visualization. Probes were visualized using ECL reagents (Pierce) and autoradiography. Developed film was scanned on a 16-bit densitometer at 300 dpi resolution and signal volume quantified using ImageQuant TL software (GE Healthcare). Protein band volumes were measured separately and then summed for comparison. In most cases no signal was observed in the RNAi lines due to silencing, in which case the same band area next to the intense band from the nontransgenic control sample was quantified, that is, background signal.

Two-Dimensional Difference Gel Electrophoresis (2D-DIGE). Total protein was isolated (*40*) and quantified with the EZQ protein quantification kit (Invitrogen). For DIGE, methanol-precipitated protein pellets were reconstituted in isoelectric focusing (IEF) media [30 mM

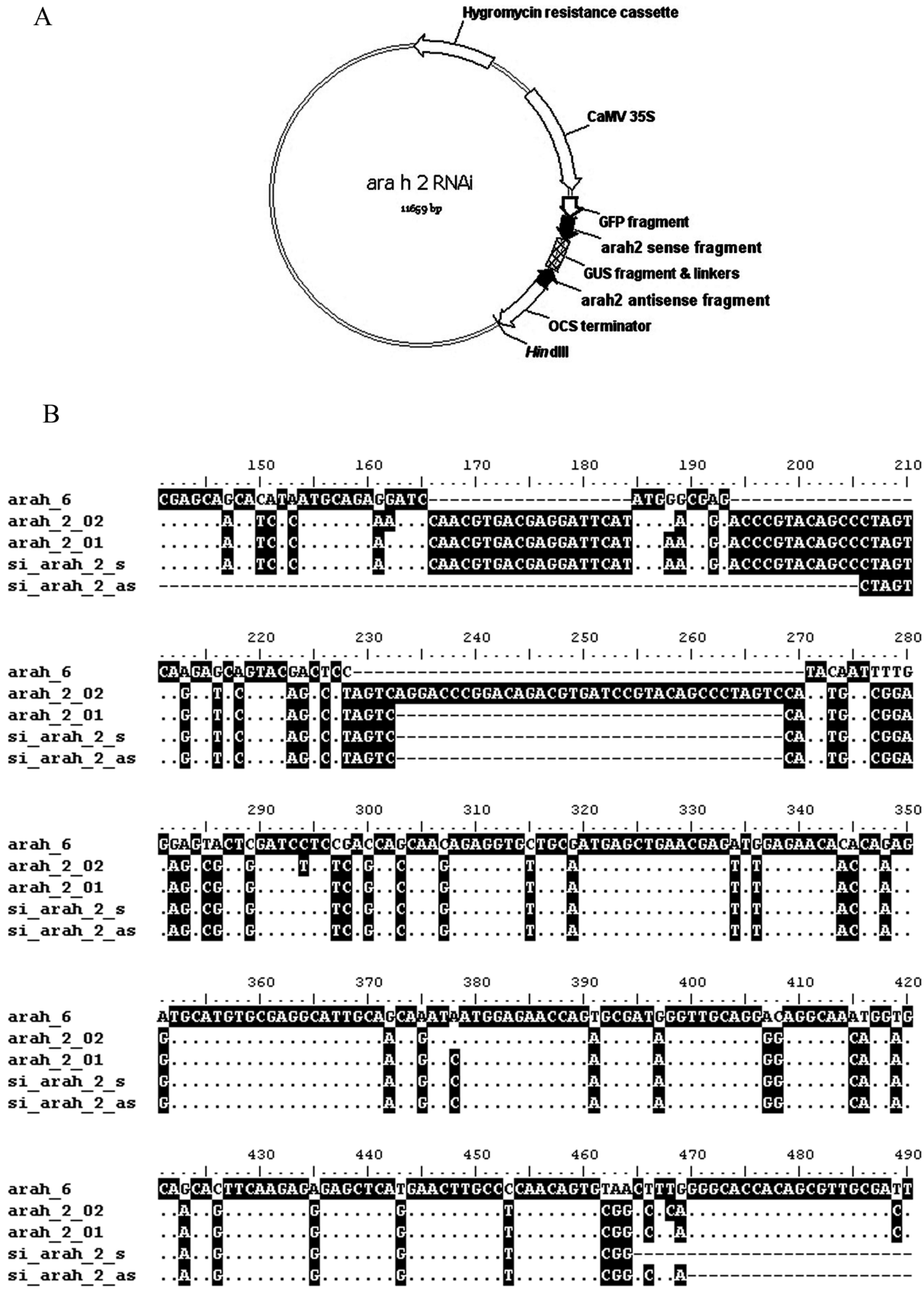


Figure 1. (**A**) *Ara h 2* RNAi construct; (**B**) alignment of nucleotide sequences of *ara h 2.01* (GenBank ID L77197), *ara h 2.02* (GenBank ID AY158467), *ara h 6* (GenBank ID AF092846), and *ara h 2* sense and antisense fragments inserted into the RNAi construct.

Tris-HCl pH 8.5, 7 M urea, 2 M thiourea, 4% (w/v) CHAPS] with gentle mixing for 30 min at room temperature followed by centrifugation for 15 min at 14000*g* to remove insoluble material. Fifty micrograms of protein was taken and adjusted to a final volume of 10 μL with IEF media. One microliter of CyDye (200 pmol) was added to protein samples and incubated on ice for 30 min in the dark. The labeling reaction was terminated with 1 μL of 10 mM lysine on ice for an additional 10 min in the dark. For IEF, 50 μg of protein was mixed with an equal volume of 2× sample buffer [8 M urea, 130 mM DTT, and 4% (w/v) CHAPS], incubated for 10 min on ice, mixed with 2.25

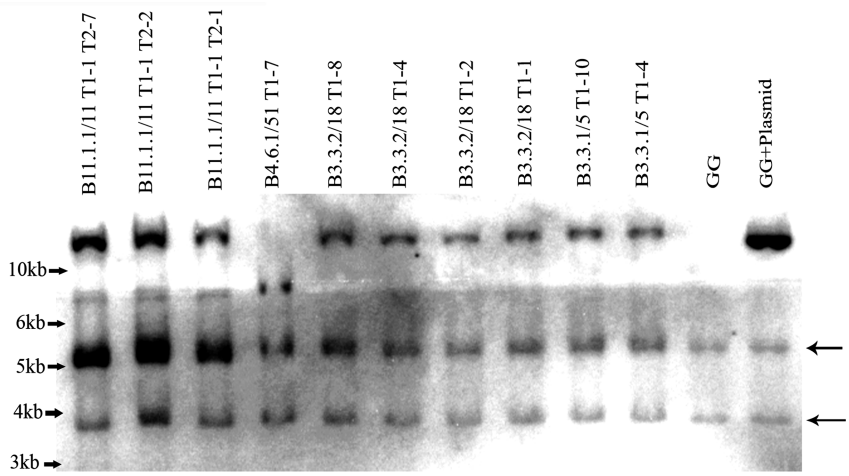


Figure 2. Southern blot of Ara h 2 silenced lines. DNA samples were digested with *Hin*dIII and hybridized with a probe specific to the *ara h 2* coding sequence. Two fragments that are present in all lanes including control represent endogenous *ara h 2* (arrow heads).

μL of IPG buffer (GE Healthcare), and adjusted to a total volume of 450 μL with sample buffer. For preparative electrophoresis, colloidal Coomassie G-250 (CBB) stained gels, protein pellets were resuspended in IEF sample extraction buffer [8 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (v/v) Triton X-100, 50 mM DTT] with gentle mixing. For preparative IEF, 1 mg of total protein was mixed with 2.25 μL of appropriate IPG buffer in a total volume of 450 μL of IEF extraction buffer.

2D-DIGE Strategy, 2-DE Statistical Analyses, and Protein Spot Identification. Segregating Ara h 2 silenced and wild-type peanut cotyledons were analyzed from two transgenic plants in a pairwise manner using either Cy3 or Cy5 for sample labeling. Both transgenic seed lines were analyzed in biological quadruplicate along with nontransgenic controls and included an equal number (two) of dye swaps to eliminate dye bias during quantification. Labeled protein samples were resolved using both p*I* 3−10 and 4−7 immobilized pH gradient strips. DIGE gels were scanned using an FLA-5000 dual photomultiplier laser scanner equipped with a Cy3/Cy5 dual emission filter (Fuji Medical, Stamford, CT). DIGE images (16 bit TIFF) were analyzed using ImageMaster 2-D Platinum software, version 5.0 (GE Healthcare). Protein abundance was expressed as relative volume according to the normalization method provided by ImageMaster software. Statistically significant, differentially expressed proteins were selected on the basis of modified Student *t*-test analysis, as developed for ImageMaster. Differentially expressed protein spots were excised from corresponding preparative CBB stained gels and trypsin digested as described previously (*40*). Liquid chromatography−tandem mass spectrometry using a ProteomeX LTQ ion trap (Thermo-Fisher, San Jose, CA) was performed as described previously (*41*).

Seed Germination Test. Weights of 40 individual mature seeds from each of two transgenic lines were measured before imbibition. All seeds were wrapped in germination paper saturated with 0.055% ethephon solution and placed in sealed plastic bags to promote uniform germination. The bags were placed in a growth chamber at 26−28 °C for 10 days. Root length was measured for each seed before transplanting to potting mix in the greenhouse. Leaf tissues from the germinated seeds were collected for DNA extraction. PCR was performed to check for the presence or absence of the transgene in the tested seedlings. Transgenic seedlings remained in the greenhouse for next generation seed production.

***A. flavus* Fungal Infection.** Mature peanut seeds were sterilized in 70% EtOH for 3 min followed by 40% Clorox for 20 min and then rinsed three times in sterile deionized water. Seed coats were removed, and cotyledons were separated and placed in a 24-well cell culture chamber (Nalge Nunc Intl., Rochester, NY). Embryo axes were excised and saved for DNA extraction. The cotyledons were further sterilized with 10% Clorox for 5 min and washed twice with sterile water before inoculation. GFP fluorescent *A. flavus* strain GFP-70 was cultured on PDA medium at 30 °C for 7 days before the spores were harvested. Spores were suspended in 9 mL of 0.01% Triton X-100 (Sigma), and the concentration of the spore suspension was adjusted to 1×10^6/mL. Cotyledons were immersed in the spore suspension and then drained of excess water. Each cotyledon was returned to its individual cell and incubated at 30 °C for 3 days. Humidity (100%) was maintained by enclosing all cell culture chambers in a covered box with three layers of wet tissue paper at the bottom. After the presence or absence of the transgene was determined by PCR for each pair of cotyledons, 10 cotyledons from transgenic and 10 cotyledons from nontransgenic segregants per line were randomly selected for measurement of fungal GFP expression. The average GFP levels were compared statistically within each line. Total protein was extracted from cotyledons and quantified as described before. Protein concentration was adjusted to 50 ng/μL. Fifty microliters of protein extracts was used to detect GFP expression at excitation and emission wavelengths of 485 and 535 nm, respectively, using a Fluorocount instrument (Perkin-Elmer, Waltham, MA). This experiment was conducted twice. Statistical analysis was performed with a Proc MIXED procedure using SAS 9.1 (SAS Institute Inc., Cary, NC) and Chi-square analysis. A P value of <0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Stable Integration of the *ara h 2* Inverted Repeat. Hygromycin-resistant embryogenic clusters were recovered after microprojectile bombardment and named according to the following system: bombardment experiment number.plate number.resistant cluster number/primary plant number (e.g., B11.1.1/11). T1 and T2 refer to the first and second generations established from seeds. Three independent transgenic events were confirmed by Southern blot analysis of *Hin*dIII digests using *ara h 2* coding sequence as the probe. Two copies of *ara h 2* were clearly distinguished in the genome of 'Georgia Green' control as well as T1 and T2 progenies of all transgenic lines tested (**Figure 2**). B3.3.1 and B3.3.2 were shown to be sublines of the same transgenic event because their Southern blot patterns were identical in all six T1 progenies tested and displayed not only the two endogenous copies of *ara h 2* but also an ∼12 kb band indicating integration of a single copy of the transgene. An independent transgenic line, B4.6.1, represented by one T1 plant on the Southern blot, also contained a single copy of the transgene in an ∼8 kb hybridizing fragment. Three T2 progenies examined from line B11.1.1 showed multiple insertions of the transgene because ∼13 and ∼7 kb bands were detected. Furthermore, the intensities of the two endogenous *ara h 2* bands were not proportional as in the 'Georgia Green' control, suggesting that at least one copy of the transgene migrates to the same position as the higher molecular weight *ara h 2*

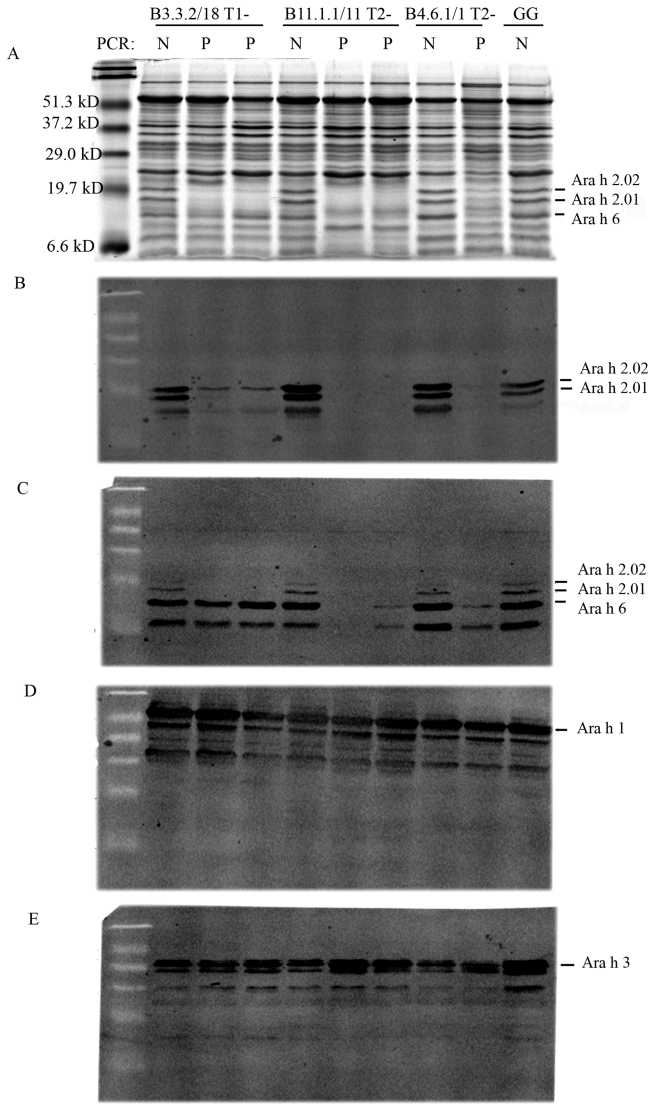


Figure 3. Western blot of Ara h 2 and Ara h 6 silenced lines. Three T1 progeny from transgenic line B3.3.2, three T2 progeny from line B11.1.1, and two T2 progeny from line B4.6.1 along with nontransgenic control 'Georgia Green' (GG) were tested. All protein samples were separated on a 15% SDS-PAGE gel and stained with (**A**) Biosafe Coomassie blue. Samples were blotted onto PVDF membranes and incubated with (**B**) anti-Ara h 2, (**C**) anti-Ara h 6, (**D**) anti-Ara h 1, and (**E**) anti-Ara h 3. Subsequently, anti-chicken secondary antibody conjugated with horseradish peroxidase was applied, and signal detection was performed with the ECL-Plus kit.

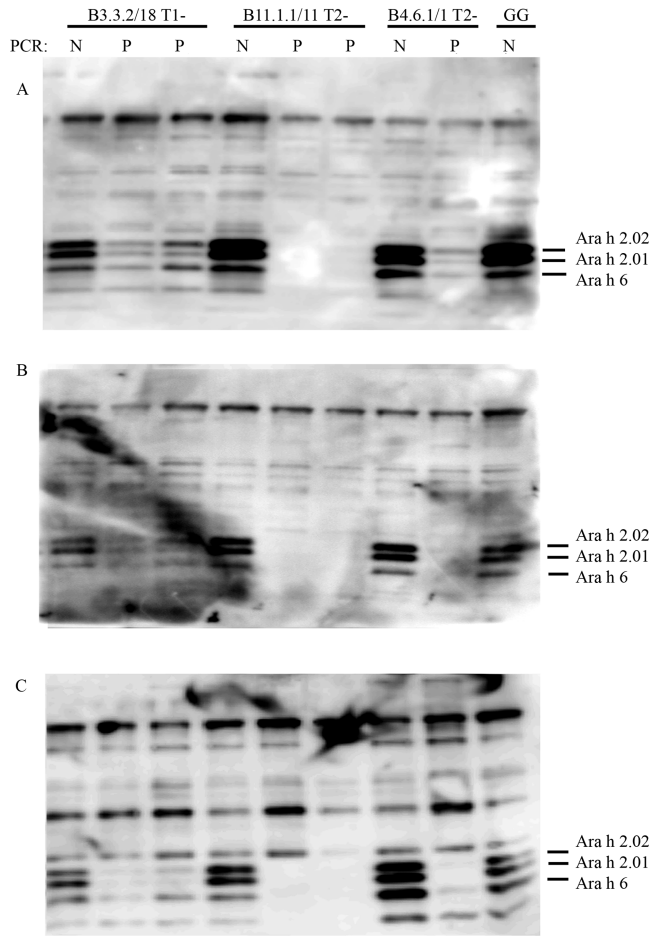
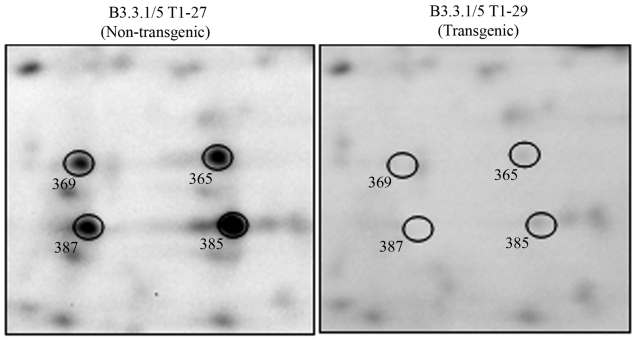


Figure 4. IgE binding of Ara h 2 and Ara h 6 silenced lines. Three T1 progeny from transgenic line B3.3.2, three T2 progeny from line B11.1.1, and two T2 progeny from line B4.6.1 along with nontransgenic control 'Georgia Green' (GG) were tested. Electrophoretically separated protein samples were blotted, and immunologically reactive fragments were detected with sera from peanut allergic patients (**A**) CM (rast = 70; screen/class = 3+; female born in 1978 with other allergy to tree nuts) (**B**) SM (rast > 100; screen/class = 2+; male born in 1965 with other allergy to tree nuts), and (**C**) DAM (rast > 100; screen/class = 4+; female born in 1972 with other allergy to tree nuts, soy, beans).

fragment in *Hin*dIII digests. Probing the Southern blot with the *gus* linker sequence revealed the same hybridization patterns as observed with the *ara h 2* gene except that no endogenous *ara h 2* was detected (data not shown). The single insertion events in lines B3.3.1-2 and B4.6.1 were confirmed, as was the presence of three fragments in line B11.1.1, including one that comigrated with the largest *ara h 2* fragment. Segregation ratios for T2 progenies of B11.1.1 and T1 progenies of B3.3.1 were 33:10 and 43:13, respectively, which are close to the expected ratio of 3:1 ($P = 0.05$) for a single dominant gene in heterozygous (hemizygous) condition. Line B4.6.1 did not produce a sufficient number of seeds for this analysis.

Silencing of Ara h 2 and Ara h 6 Results in a Reduction of IgE Binding. The nucleotide sequence alignment in **Figure 1B** shows that the *ara h 2* inverted repeat shares 81% sequence homology with the *ara h 6* coding region from position 278 to 461 (184 bp in length). To test for knockdown of these allergens, Western blots were performed on the three transgenic lines (**Figure 3**). The Coomassie Blue stained gel (**Figure 3A**) showed that global protein profiles were not notably affected by transformation with the *ara h 2* silencing construct. The Ara h 2 double bands around the 19.7 kDa marker in the PCR-positive transgenic seeds were either absent or much fainter than in the PCR-negative, nontransgenic controls. A Western blot with anti-Ara h 2 antibody showed that both Ara h 2.01 and Ara h 2.02 levels in B3.3.2 and B4.6.1 were greatly reduced but still detectable (**Figure 3B**). The expression of Ara h 2.01 and Ara h 2.02 was not detectable in line B11.1.1. The lowest molecular weight immunologically detectable band probably represents a proteolytically processed form of Ara h 2. The antibody against Ara h 6 detected four bands in nontransgenic controls (**Figure 3C**). The two faint bands around 19.7 kDa were concluded to be Ara h 2.01 and Ara h 2.02 because the signal at these positions was reduced in all transgenic seeds. Ara h 6 levels in B3.3.2 were not substantially different from nontransgenic control seeds, indicating that Ara h 2 is the primary knockdown target in this line. Ara h 6 levels in B11.1.1 and B4.6.1, however, were greatly reduced; therefore, silencing



spot	$\log_2$ ratio	protein identification	score	% coverage	# peptides
365	3.7	allergen Ara h 2.02	20.2	9.9	2
369	2.9	allergen Ara h 2.02	20.1	12.2	2
385	4.0	allergen Ara h 2.02	14.1	11.6	2
387	3.9	allergen Ara h 2.02	48.2	21.5	6

Figure 5. 2D-DIGE of T1 seeds from Ara h 2 silenced line B3.3.1/5. The 2D images were cropped from the same area of transgenic and nontransgenic samples. The four prominent Ara h 2 protein spots 365, 369, 385, and 387 are noted on the respective gels. Spot volume expression data ($\log_2$(nontransgenic/transgenic) and corresponding mass spectrometry identification information including protein identification, score (a composite of SEQUEST cross-correlation peptide values), percent protein coverage, and number of identified peptides (unique and nonoverlapping peptides) are shown.

of both Ara h 2 and Ara h 6 was accomplished in these two transgenic lines. Two more peanut allergens, Ara h 1 and Ara h 3, also were tested by Western blot analysis (**Figures 3D**,**E**). The expression of these two allergens was not detectably affected by Ara h 2 silencing.

The same protein extracts from all three transgenic lines were tested for IgE binding capacity using peanut allergic patient sera (**Figure 4**). All three patients recognize multiple peanut allergens including Ara h 2 and Ara h 6. Ara h 2.01 and Ara h 2.02 in transgenic subline B3.3.2 could be detected by sera from all three patients but at greatly reduced levels compared to the nontransgenic control. Neither Ara h 2 nor Ara h 6 in transgenic line B11.1.1 was detectable by IgE binding, and the levels of these two proteins were greatly reduced in line B4.6.1. These data were highly consistent with the Western blot data obtained using antigen specific antibodies raised from chicken.

Quantitative 2D-DIGE Analyses of Ara h 2 Silencing. Silencing of Ara h 2 was confirmed by quantitative 2D-DIGE using T1 seeds from sublines B3.3.1 and B3.3.2. Comprehensive 2D-DIGE analysis of both sublines revealed a single cluster of 2-DE spots that were consistently differential. Four protein spots, 365, 369, 385, and 387, with apparent masses between 16 and 23 kDa were reduced on average 13-, 7.2-, 16-, and 15-fold, respectively, in transgenic subline B3.3.1 compared to nontransgenic controls (**Figure 5**). Corresponding spots 458, 459, 477, and 476 in transgenic subline B3.3.2 were reduced 17-, 14-, 19-, and 15-fold, respectively, compared to nontransgenic controls. Liquid chromatography−tandem mass spectrometry analysis of tryptic peptides from these protein spots revealed each of these four spots mapped to Ara h 2.02 (GenBank accession no. 26245447). For each of these protein assignments the number of unique, nonoverlapping tryptic peptides was two or greater. Additionally, non-Ara h 2 protein spots were found to be differentially expressed, and they will be verified and presented in a follow-up study.

Quantitative Immunoblotting Demonstrates Complete Silencing of Ara h 2 and Ara h 6 in Mature Cotyledons of Line 11.1.1. To further quantify Ara h 2 and Ara h 6 silencing, quantitative immunoblotting was performed on total proteins

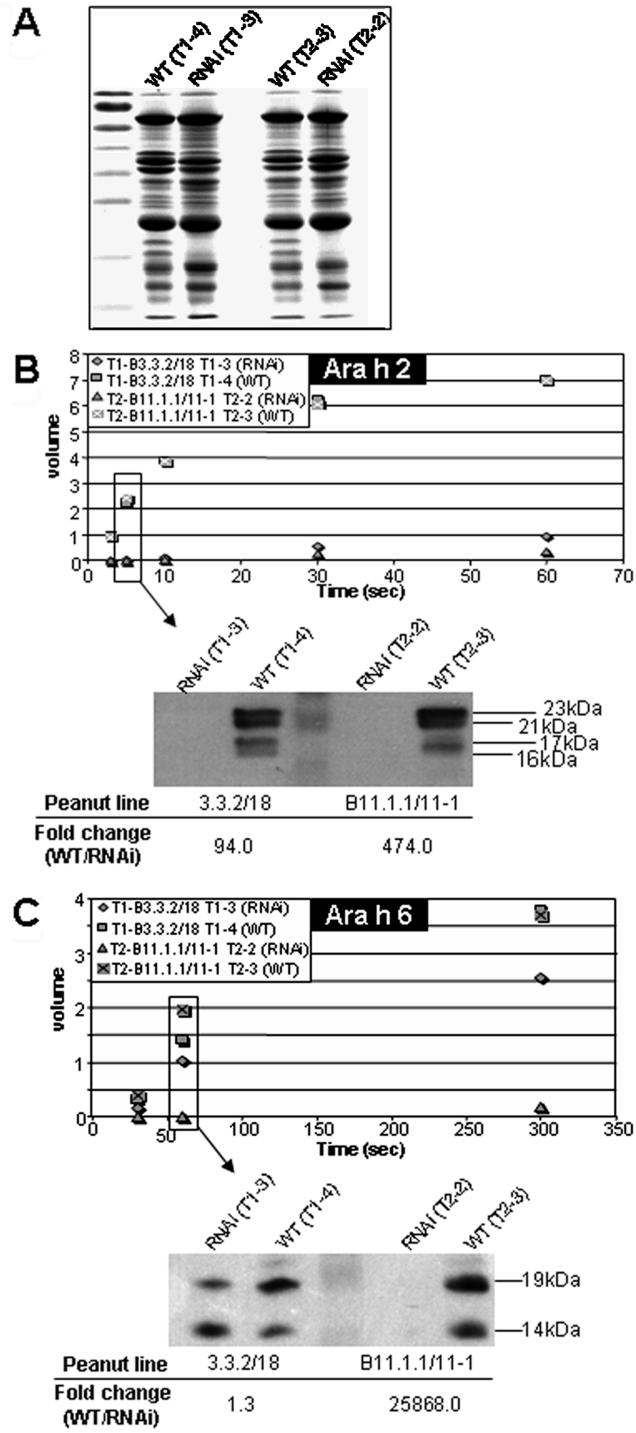


Figure 6. Quantitative immunoblotting to verify complete silencing of Ara h 2 and Ara h 6 in transgenic lines. Equal amounts of protein (20 μg) from nontransgenic control and RNAi Ara h 2 lines were resolved by SDS-PAGE and stained by colloidal Coomassie Brilliant Blue (**A**) or transferred to PVDF and immunostained with antibodies to either Ara h 2 (**B**) or Ara h 6 (**C**). Chemilluminescent signal was quantified by autoradiography followed by scanning densitometry and analysis by ImageQuantTL software. Signal volume was summed for all bands shown in lower panels and plotted versus development time to determine the linear range of detection. In both cases the early time points were within the linear range, and the band volume ratio of nontransgenic (denoted wild type, WT) to transgenic (for indicated time point) is shown for two RNAi lines.

extracted from mature cotyledons (**Figure 6**). Protein (20 μg) from nontransgenic and transgenic lines was resolved by SDS-

Table 1. Seed Weight and Seedling Root Length of Ara h 2- and Ara h 6-Silenced Lines

line	PCR	seed no.[a]	seed wt (mg)	*P* value	root length (cm)	*P* value
B3.3.1/5 T1-1 T2- T3-	transgenic	17	346.1 ± 20.6	0.21	2.85 ± 0.18	0.62
	nontransgenic	21	387.4 ± 23.6		3.03 ± 0.21	
B11.1.1/11 T1- T2-	transgenic	25	325.6 ± 21.2	0.33	3.52 ± 0.24	0.72
	nontransgenic	14	358.0 ± 21.7		3.81 ± 0.27	

[a] Forty seeds per line were evaluated in a single experiment. Radicle extension was measured 10 days after imbibition on germination paper. Three seeds germinated according to radicle extension but failed to survive after transplanting into soil; therefore, their identities as transgenic or nontransgenic were not available, and their data were not included.

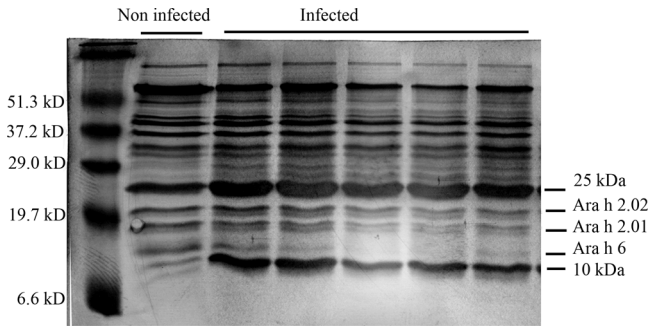


Figure 7. Ara h 2 is not preferentially degraded by *Aspergillus flavus*. 'Georgia Green' seeds were infected with *A. flavus* for 10 days. Proteins extracted from noninfected and infected seeds were separated on a 15% SDS-PAGE gel and stained with Coomassie Blue. The enhancement of 10 and 25 kDa protein bands is caused by fungal infection. The Ara h 2 double bands and Ara h 6 were not preferentially degraded as compared to the other peanut proteins.

PAGE, blotted to PVDF, and probed with Ara h 2 and Ara h 6 antibodies for chemiluminescence development. Coomassie staining verified equal loading of mature cotyledon proteins (**Figure 6A**), and antibodies to Ara h 2 revealed four protein bands of 16, 17, 21, and 23 kDa, in agreement with the masses of the four Ara h 2 protein spots observed by 2D-DIGE (**Figure 5**). Because the identity of each of these proteins was verified by mass spectrometry, the band volumes were summed for quantitative analysis (**Figure 6B**). Kinetic analysis of the signal for the sum of these four bands revealed the first two time points were within the linear range of signal detection. The results of the 5 s time point showed Ara h 2 was 94- and 474-fold reduced in lines B3.3.2 and B11.1.1, respectively. Antibodies to Ara h 6 detected protein bands at 14 and 19 kDa (**Figure 6C**). At the 60 s time point Ara h 6 was 1.3- and 25868-fold reduced in lines B3.3.2 and B11.1.1, respectively. It can be concluded that Ara h 2 and Ara h 6 are both completely silenced in line B11.1.1.

To achieve the purpose of down-regulating Ara h 2 and Ara h 6 simultaneously, the transgene construct was designed to include a portion of the coding region of *ara h 2* that shares 81% sequence homology to *ara h 6*, arranged in an inverted repeat to facilitate hairpin RNA formation. The dsRNA presumably is processed into 21−24 nucleotide siRNAs by endogenous Dicer proteins. These siRNAs can be recruited into an RNA-induced silencing complex to guide the degradation of target mRNA containing complementary sequences (*42*). Previously, both of the tomato allergen isoforms Lyc e 1.01 and Lyc e 1.02 were silenced by an inverted repeat transgene construct targeting gene regions with 84.4% sequence similarity (*30*). Similarly Lyc e 3.01 and Lyc e 3.02 with 76.5% sequence homology were also silenced by one RNAi construct (*31*).

Silencing abundant seed storage proteins such as Ara h 2 and Ara h 6 has been questioned due to concerns for peanut plant health and seed quality (*43*). Our study demonstrated that Ara h 2 and Ara h 6 silencing was sequence specific with few changes in other abundant proteins as detected by Coomassie blue staining including peanut allergens Ara h 1 and Ara h 3 as detected by Western blot. SDS-PAGE and Coomassie blue staining detected only abundant proteins, whereas 2D-DIGE is a more sensitive method that can detect and resolve low-abundance proteins. A strong reduction in four Ara h 2 protein species was confirmed by 2D-DIGE analyses, a global proteomics approach. Quantitative immunoblotting conclusively demonstrated that Ara h 2 and Ara h 6 were completely silenced in line B11.1.1. Furthermore, IgE binding from sera of three peanut patients demonstrated distinctive recognition patterns to multiple allergens. Other than the reduction in Ara h 2 and Ara h 6 binding, overall IgE binding patterns were consistent between the transgenic and nontransgenic controls. Therefore, silencing of Ara h 2 and Ara h 6 had little apparent collateral effect on allergen detection by patient sera, although minor effects on other proteins are being further investigated (unpublished results). Previously, silencing Gly m Bd 30K, a soybean allergen, also demonstrated target specificity of RNAi, and no significant differences were observed between the transgenic and nontransgenic lines as determined by proteomic analysis (*27*). Our results are in contrast, however, to an earlier report of Ara h 2 silencing (*44*), where major differences in seed protein patterns were reported.

The level of reduction in IgE binding to Ara h 2 and Ara h 6 may not be proportional to the reduction of allergenicity. The criteria of allergenicity not only include IgE binding but also the ability of the allergen to cross-link IgE-associated effector cells and instigate allergic responses in susceptible patients. Complete removal of Ara h 2 by immunoprecipitation had only minor impact on peanut allergenicity (20% reduction in potency) (*45*). The investigators concluded that peanut allergens other than Ara h 2 accounted for the major effectors of allergenicity. Similarly, in our study, serum IgE from all three patients recognized other peanut allergens besides Ara h 2. It is unlikely that our Ara h 2 and Ara h 6 silenced peanut could mitigate their allergic response. Admittedly, the majority of patient IgE binding patterns are complex and involve multiple peanut allergens; however, a subset of peanut allergic patients was reported to have serum IgE specifically recognizing Ara h 2 and Ara h 6. Studies have found that 12 of 32 (37.5%) peanut allergic patients from the Netherlands reacted only to Ara h 2 and/or Ara h 6 (*14, 46*). Similarly, 9 of 40 (22.5%) patients from the United Kingdom were found to have IgE that specifically recognizes Ara h 2 and/or Ara h 6 (*47*). Silencing of tomato allergens Lyc e 1.01 and Lyc e 1.02 reduced the wheal size by 63−100% in patients whose IgE bound exclusively to Lyc e 1, whereas only a 16−25% reduction in wheal size was found in patients whose IgE recognized multiple allergens in addition to Lyc e 1 (*30*). Therefore, Ara h 2- and Ara h 6-silenced peanut potentially could be consumed by those 22.5−37.5% of patients without triggering the allergic reaction. Also, because Ara h 2 is the least abundant of the major allergens, and yet has previously been shown to be the most

potent peanut allergen, it is not unreasonable to believe that the severity of the allergic reaction for other allergic individuals would be reduced or the threshold of reactivity increased. It is also possible that fewer individuals may become sensitized to a peanut missing its most potent allergenic protein. For the general peanut allergic population, eliminating three to four major peanut allergens still would be necessary; however, the feasibility of silencing all major peanut allergens is debatable and subject to future experimental results.

Ara h 2 and Ara h 6 Knockdown Does Not Affect Seedling Growth nor Does It Promote Fungal Growth. Two lines, B3.3.1 and B11.1.1, were tested for seed weight and seed germination. The mean value of transgenic seed weight was slightly less than that of the nontransgenic control; however, the difference did not reach statistical significance (**Table 1**). There also was no significant difference in radicle lengths between transgenic and nontransgenic seedlings (**Table 1**). At plant maturity, no morphological differences were observed.

The transcription of *ara h 2* and *ara h 6* appears to be seed specific and cannot be detected in other peanut organs such as flower, peg, leaf, and root (*15, 16*). Seed developmental stages 2, 3, and 4 accumulate high levels of *ara h 2* and *ara h 6* transcripts. Ara h 2 and Ara h 6 proteins reach their highest levels in mature seeds and are degraded during seedling germination (*16*). It is therefore conceivable that a reduction in Ara h 2 and Ara h 6 proteins could affect peanut seed traits. However, we observed no significant morphological changes between the transgenic and nontransgenic plants segregating from a transgenic line. Data on seed weight and germination support that silencing of both Ara h 2 and Ara h 6 does not affect the vital physiological function of seeds.

Cotyledons of 'Georgia Green' peanut seeds were inoculated with spores from *A. flavus*. Mobilization of Ara h 2 and Ara h 6 to support *Aspergillus* fungal growth was evaluated 10 days after inoculation. SDS-PAGE of molded peanut protein extracts shows clear enhancement of 10 and 25 kDa protein bands as compared to the noninfected control (**Figure 7**). These proteins could be fungal proteins or peanut seed protein profile changes as a consequence of fungal infection. Ara h 2.01, Ara h 2.02, and Ara h 6 in the infected seeds decreased slightly, but there did not appear to be preferential degradation of these proteins upon fungal infection. Fungal growth after 3 days of infection was measured by GFP expression in the GFP-70 strain. The data show no significant differences in fungal growth between the transgenic (relative fluorescence units of 10188.8 ± 1125.9 and 12882.1 ± 2630.5) and nontransgenic (RFU of 12890.5 ± 1122.5 and 12564.2 ± 931.2) seeds of lines B3.3.1 and B11.1.1, respectively. A trend of fungal growth suppression in line B3.3.1 was observed.

Functionally, native Ara h 2 was found to act as a weak trypsin inhibitor that protects Ara h 1 from degradation in the presence of trypsin enzyme (*18*). Trypsin inhibitors in other crops have been characterized as part of the defense mechanism against insects and pathogens (*19, 21*). *A. flavus* contamination is a major concern for the peanut industry due to the strong carcinogenic potency of its metabolite, aflatoxin. Knocking down Ara h 2 expression in peanut potentially could increase its susceptibility to *Aspergillus* fungal infection and aflatoxin production, a consequence conflicting with the goal of allergen reduction. As a precedent, silencing a 14−16 kDa trypsin inhibitor in maize increases *A. flavus* colonization and aflatoxin production (*22*). In our study, Ara h 2 and Ara h 6 were not preferentially utilized to support fungal growth in vitro, and silenced peanut transgenic lines do not promote *A. flavus* fungal growth. The correlation between in vitro fungal colonization and field aflatoxin contamination is weak, however. Several peanut genotypes showing in vitro aflatoxin resistance did not demonstrate any resistance in the field (*48*). Environmental factors such as drought and heat contribute significantly to the production of aflatoxin. Therefore, the effect of Ara h 2 and Ara h 6 silencing on aflatoxin production needs to be further tested by field study.

Transgenic silencing of Ara h 2 and Ara h 6 may not be directly acceptable for commercial production due to the stringent regulation of genetically modified crops and potential instability of RNAi. However, the current study provides useful information to investigate the functional role of Ara h 2 and Ara h 6 in peanut and demonstrates that knocking out Ara h 2 and Ara h 6 through mutagenesis could be pursued as a stable alternative to RNAi (*26*).

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LITERATURE CITED

(1) Sicherer, S. H.; Munoz-Furlong, A.; Sampson, H. A. Prevalence of peanut and tree nut allergy in the United States determined by means of a random digit dial telephone survey: a 5-year follow-up study. *J. Allergy Clin. Immunol.* **2003**, *112*, 1203–1207.

(2) Grundy, J.; Matthews, S.; Bateman, B.; Dean, T.; Arshad, S. H. Rising prevalence of allergy to peanut in children: Data from 2 sequential cohorts. *J. Allergy Clin. Immunol.* **2002**, *110*, 784–789.

(3) Burks, W. Peanut allergy: a growing phenomenon. *J. Clin. Invest.* **2003**, *111*, 950–952.

(4) Wensing, M.; Penninks, A. H.; Hefle, S. L.; Koppelman, S. J.; Bruijnzeel-Koomen, C. A.; Knulst, A. C. The distribution of individual threshold doses eliciting allergic reactions in a population with peanut allergy. *J. Allergy Clin. Immunol.* **2002**, *110*, 915–920.

(5) Al-Muhsen, S.; Clarke, A. E.; Kagan, R. S. Peanut allergy: an overview. *Can. Med. Assoc. J.* **2003**, *168*, 1279–1285.

(6) Fleischer, D. M.; Conover-Walker, M. K.; Christie, L.; Burks, A. W.; Wood, R. A. Peanut allergy: recurrence and its management. *J. Allergy Clin. Immunol.* **2004**, *114*, 1195–1201.

(7) Pons, L.; Chery, C.; Romano, A.; Namour, F.; Artesani, M. C.; Gueant, J. L. The 18 kDa peanut oleosin is a candidate allergen for IgE-mediated reactions to peanuts. *Allergy* **2002**, *57*, 88–93.

(8) de Leon, M. P.; Rolland, J. M.; O'Hehir, R. E. The peanut allergy epidemic: allergen molecular characterisation and prospects for specific therapy. *Expert Rev. Mol. Med* **2007**, *9*, 1–18.

(9) Flinterman, A. E.; van Hoffen, E.; den Hartog Jager, C. F.; Koppelman, S.; Pasmans, S. G.; Hoekstra, M. O.; Bruijnzeel-Koomen, C. A.; Knulst, A. C.; Knol, E. F. Children with peanut allergy recognize predominantly Ara h2 and Ara h6, which remains stable over time. *Clin. Exp. Allergy* **2007**, *37*, 1221–1228.

(10) Burks, A. W.; Cockrell, G.; Connaughton, C.; Karpas, A.; Helm, R. M. Epitope specificity of the major peanut allergen, Ara h II. *J. Allergy Clin. Immunol.* **1995**, *95*, 607–611.

(11) Suhr, M.; Wicklein, D.; Lepp, U.; Becker, W. M. Isolation and characterization of natural Ara h 6: evidence for a further peanut allergen with putative clinical relevance based on resistance to pepsin digestion and heat. *Mol. Nutr. Food Res.* **2004**, *48*, 390–399.

(12) Lehmann, K.; Schweimer, K.; Reese, G.; Randow, S.; Suhr, M.; Becker, W. M.; Vieths, S.; Rosch, P. Structure and stability of 2S albumin-type peanut allergens: implications for the severity of peanut allergic reactions. *Biochem. J.* **2006**, *395*, 463–472.

(13) Ramos, M. L.; Fleming, G.; Chu, Y.; Akiyama, Y.; Gallo, M.; Ozias-Akins, P. Chromosomal and phylogenetic context for conglutin genes in *Arachis* based on genomic sequence. *Mol. Genet. Genomics* **2006**, *275*, 578–592.

(14) Koppelman, S. J.; de Jong, G. A.; Laaper-Ertmann, M.; Peeters, K. A.; Knulst, A. C.; Hefle, S. L.; Knol, E. F. Purification and immunoglobulin E-binding properties of peanut allergen Ara h

6: evidence for cross-reactivity with Ara h 2. *Clin. Exp. Allergy* **2005**, *35*, 490–497.

(15) Paik-Ro, O. G.; Seib, J. C.; Smith, R. L. Seed-specific, developmentally regulated genes of peanut. *Theor. Appl. Genet.* **2002**, *104*, 236–240.

(16) Kang, I. H.; Srivastava, P.; Ozias-Akins, P.; Gallo, M. Temporal and spatial expression of the major allergens in developing and germinating peanut seed. *Plant Physiol.* **2007**, *144*, 836–845.

(17) Chatel, J. M.; Bernard, H.; Orson, F. M. Isolation and characterization of two complete Ara h 2 isoforms cDNA. *Int. Arch. Allergy Immunol.* **2003**, *131*, 14–18.

(18) Maleki, S. J.; Viquez, O.; Jacks, T.; Dodo, H.; Champagne, E. T.; Chung, S. Y.; Landry, S. J. The major peanut allergen, Ara h 2, functions as a trypsin inhibitor, and roasting enhances this function. *J. Allergy Clin. Immunol.* **2003**, *112*, 190–195.

(19) Ryan, C. A. Protease inhibitors in plants: genes for improving defenses against insects and pathogens. *Annu. Rev. Phytopathol.* **1990**, *28*, 425–449.

(20) Hermosa, M. R.; Turrà, D.; Fogliano, V.; Monte, E.; Lorito, M. Identification and characterization of potato protease inhibitors able to inhibit pathogenicity and growth of *Botrytis cinerea*. *Physiol. Mil. Plant Pathol.* **2006**, *68*, 138–148.

(21) Chen, Z. Y.; Brown, R. L.; Damann, K. E.; Cleveland, T. E. Identification of maize kernel endosperm proteins associated with resistance to aflatoxin contamination by *Aspergillus flavus*. *Phytopathology* **2007**, *97*, 1094–1103.

(22) Chen, Z. Y.; Brown, R. L.; Cleveland, T. E.; Damann, K. E. The expression of maize 14 kDa trypsin inhibitor protein on host resistance to *Aspergillus flavus* infection and aflatoxin production. *Phytopathology* **2008**, *98*, S35.

(23) Scurlock, A. M.; Burks, A. W. Peanut allergenicity. *Ann. Allergy Asthma Immunol.* **2004**, *93*, S12–18.

(24) Rabjohn, P.; West, C. M.; Connaughton, C.; Sampson, H. A.; Helm, R. M.; Burks, A. W.; Bannon, G. A. Modification of peanut allergen Ara h 3: effects on IgE binding and T cell stimulation. *Int. Arch. Allergy Immunol.* **2002**, *128*, 15–23.

(25) Li, X. M.; Srivastava, K.; Grishin, A.; Huang, C. K.; Schofield, B.; Burks, W.; Sampson, H. A. Persistent protective effect of heat-killed *Escherichia coli* producing "engineered," recombinant peanut proteins in a murine model of peanut allergy. *J. Allergy Clin. Immunol.* **2003**, *112*, 159–167.

(26) Ozias-Akins, P.; Ramos, M. L.; Chu, Y., Hypoallergenic foods beyond infant formulas. In *Food Allergy*; Maleki, S. J., Burks, A. W., Helm, R. M., Eds.; ASM Press: Washington, DC, 2006; pp 287−306.

(27) Herman, E. M.; Helm, R. M.; Jung, R.; Kinney, A. J. Genetic modification removes an immunodominant allergen from soybean. *Plant Physiol.* **2003**, *132*, 36–43.

(28) Tada, Y.; Nakase, M.; Adachi, T.; Nakamura, R.; Shimada, H.; Takahashi, M.; Fujimura, T.; Matsuda, T. Reduction of 14−16 kDa allergenic proteins in transgenic rice plants by antisense gene. *FEBS Lett.* **1996**, *391*, 341–345.

(29) Gilissen, L. J.; Bolhaar, S. T.; Matos, C. I.; Rouwendal, G. J.; Boone, M. J.; Krens, F. A.; Zuidmeer, L.; Van Leeuwen, A.; Akkerdaas, J.; Hoffmann-Sommergruber, K.; Knulst, A. C.; Bosch, D.; Van de Weg, W. E.; Van Ree, R. Silencing the major apple allergen Mal d 1 by using the RNA interference approach. *J. Allergy Clin. Immunol.* **2005**, *115*, 364–369.

(30) Le, L. Q.; Mahler, V.; Lorenz, Y.; Scheurer, S.; Biemelt, S.; Vieths, S.; Sonnewald, U. Reduced allergenicity of tomato fruits harvested from Lyc e 1-silenced transgenic tomato plants. *J. Allergy Clin. Immunol.* **2006**, *118*, 1176–1183.

(31) Le, L. Q.; Lorenz, Y.; Scheurer, S.; Fotisch, K.; Enrique, E.; Bartra, J.; Biemelt, S.; Vieths, S.; Sonnewald, U. Design of tomato fruits with reduced allergenicity by dsRNAi-mediated inhibition of ns-LTP (Lyc e 3) expression. *Plant Biotechnol. J.* **2006**, *4*, 231–242.

(32) Bhalla, P. L.; Swoboda, I.; Singh, M. B. Antisense-mediated silencing of a gene encoding a major ryegrass pollen allergen. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 11676–11680.

(33) Samoylov, V. M.; Tucker, D. M.; Parrott, W. A. Soybean [*Glycine max* (L.) Merill] embryogenic cultures: The role of sucrose and total nitrogen content on proliferation. *In Vitro Cell Dev. Biol. Plant* **1998**, *34*, 8–13.

(34) Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol. Plant.* **1962**, *15*, 473–497.

(35) Gamborg, O. L.; Miller, R. A.; Ojima, K. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* **1968**, *50*, 150–158.

(36) Ozias-Akins, P.; Schnall, J. A.; Anderson, W. F.; Singsit, C.; Clemente, T.; Adang, M. J.; Weissinger, A. K. Regeneration of transgenic peanut plants from stably transformed embryogenic callus. *Plant Sci.* **1993**, *93*, 185–194.

(37) Singsit, C.; Adang, M. J.; Lynch, R. E.; Anderson, W. F.; Wang, A.; Cardineau, G.; Ozias-Akins, P. Expression of a *Bacillus thuringiensis* cryIA(c) gene in transgenic peanut plants and its efficacy against lesser cornstalk borer. *Transgenic Res.* **1997**, *6*, 169–176.

(38) Sharma, A. D.; Gill, P. K.; Singh, P. DNA isolation from dry and fresh samples of polysaccharide-rich plants. *Plant Mol. Biol. Rep.* **2002**, *20*, 415a–415f.

(39) Chen, S.; Dickman, M. B. Bcl-2 family members localize to tobacco chloroplasts and inhibit programmed cell death induced by chloroplast-targeted herbicides. *J. Exp. Bot.* **2004**, *55*, 2617–2623.

(40) Hajduch, M.; Ganapathy, A.; Stein, J. W.; Thelen, J. J. A systematic proteomic study of seed filling in soybean. Establishment of high-resolution two-dimensional reference maps, expression profiles, and an interactive proteome database. *Plant Physiol.* **2005**, *137*, 1397–1419.

(41) Hajduch, M.; Casteel, J. E.; Hurrelmeyer, K. E.; Song, Z.; Agrawal, G. K.; Thelen, J. J. Proteomic analysis of seed filling in *Brassica napus*: developmental characterization of metabolic isozymes using high-resolution two-dimensional gel electrophoresis. *Plant Physiol.* **2006**, *141*, 32–46.

(42) Small, I. RNAi for revealing and engineering plant gene functions. *Curr. Opin. Biotechnol.* **2007**, *18*, 148–153.

(43) Burks, A. W. Peanut allergy. *Lancet* **2008**, *371*, 1538–15346.

(44) Dodo, H. W.; Konan, K. N.; Chen, F. C.; Egnin, M.; Viquez, O. M. 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. *Plant Biotechnol. J.* **2008**, *6*, 135–145.

(45) McDermott, R. A.; Porterfield, H. S.; El Mezayen, R.; Burks, A. W.; Pons, L.; Schlichting, D. G.; Solomon, B.; Redzic, J. S.; Harbeck, R. J.; Duncan, M. W.; Hansen, K. C.; Dreskin, S. C. Contribution of Ara h 2 to peanut-specific, immunoglobulin E-mediated, cell activation. *Clin. Exp. Allergy* **2007**, *37*, 752–763.

(46) Koppelman, S. J.; Wensing, M.; Ertmann, M.; Knulst, A. C.; Knol, E. F. Relevance of Ara h1, Ara h2 and Ara h3 in peanut-allergic patients, as determined by immunoglobulin E Western blotting, basophil-histamine release and intracutaneous testing: Ara h2 is the most important peanut allergen. *Clin. Exp. Allergy* **2004**, *34*, 583–590.

(47) Kleber-Janke, T.; Crameri, R.; Appenzeller, U.; Schlaak, M.; Becker, W. M. Selective cloning of peanut allergens, including profilin and 2S albumins, by phage display technology. *Int. Arch. Allergy Immunol.* **1999**, *119*, 265–274.

(48) Anderson, W. F.; Holbrook, C. C.; Wilson, D. M.; Matheron, M. E. Evaluation of preharvest aflatoxin contamination in several potentially resistant peanut genotypes. *Peanut Sci.* **1995**, *22*, 29–32.

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