

Skin prick tests reveal stable and heritable reduction of allergenic potency of gene-silenced tomato fruits

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Background: Today, for patients with food allergy, the only possibility to prevent allergic reactions is avoidance of the allergenic food. Genetic engineering of hypoallergenic plants by means of RNA interference (RNAi) could be an approach to improve the quality of life of subjects with food allergy.

Objectives: We sought to achieve stable inhibition of expression of the allergenic nonspecific lipid transfer protein *Lyc e 3* in tomato and to analyze the reduction of allergenicity *in vitro* by using histamine release assays and *in vivo* by using skin prick tests with transgenic tomato fruits.

Methods: Gene silencing was performed by means of RNAi and monitored by using Western blotting with nonspecific lipid transfer protein-specific antibodies and sera from patients with tomato allergy. Dose-dependent basophil histamine release assays, prick-to-prick skin testing, and determination of endogenous histamine content were performed with fruits harvested from plants of the first and second generation to assess the allergenic potency compared with that of wild-type fruits.

Results: We demonstrated that silencing of *Lyc e 3* by means of RNAi contributes to reduced skin reactivity and is passed on to the next generation of fruits. A significant reduction of allergenic potency was determined *in vitro* and confirmed by using skin prick tests.

Conclusion: Taken together, these results indicate that RNAi technology is an effective tool to generate foods with reduced allergenicity.

Clinical implications: Allergen-reduced plant foods might allow reduction of dietary restrictions for patients allergic to panallergen families. (*J Allergy Clin Immunol* ■■■■;■■■:■■■-■■■.)

Key words: Gene silencing, RNA interference, food allergy, tomato, *Lyc e 3*, allergenic potency, hypoallergenic, genetically modified food

Abbreviations used

AP: Alkaline phosphatase
LTP: Lipid transfer protein
nsLTP: Nonspecific lipid transfer protein
RNAi: RNA interference
SPT: Skin prick test
T0: First generation
T1: Second generation

Food allergies represent an increasing health issue. Generating foods with reduced allergenicity by genetic modification could provide an alternative to food avoidance and improve the quality of life of patients with food allergy. Initial attempts focused on downregulating expression of allergenic proteins, such as a 15- to 16-kD protein from rice¹ and Gly m Bd 30k from soybean,² by using antisense approaches. The authors were able to show a significant reduction of IgG and IgE binding using *in vitro* tests. In the meantime, RNA interference (RNAi) with double-stranded short-interfering RNA for posttranscriptional gene silencing has been shown to be a very efficient strategy for inhibition of protein expression in plants. Recently, the RNAi technology was applied to suppress the expression of the major apple allergen Mal d 1³ and Ara h 2 in peanuts.⁴ Preliminary data showed suppression of Ara h 2 in plantlets, as identified by means of PCR and Southern and Northern blotting. Moreover, suppression of Mal d 1 was analyzed by using IgE and IgG immunoblotting. All transformed apple plants showed weak or no reactivity with a specific antibody or pooled patients' sera, and the leaves from transgenic plants showed a reduced allergenic potency *in vivo* by skin prick tests (SPTs) in 3 patients. Recently, we have shown that 2 isoforms of a newly identified tomato allergen, *Lyc e 3*, can be stably suppressed in leaves, as well as in fruits.⁵ Silencing of both isoforms by means of RNAi technology showed an almost complete suppression of mRNA coding for nonspecific lipid transfer protein (nsLTP) and protein expression in the transgenic tomato (*Solanum esculentum*) plants, including fruits.⁵

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Tomato has been reported as a food that causes allergic reactions after ingestion in patients with food allergy.^{6,7} Profilin, β -fructofuranosidase, and nsLTP were recently identified as allergens in tomato^{5,8-11} and denominated as Lyc e 1, Lyc e 2, and Lyc e 3, respectively, by the International Union of Immunological Societies Allergen Nomenclature Subcommittee. Lyc e 3 belongs to a family of nsLTPs with a molecular size of 8 to 10 kd and is a member of the group of 14 pathogenesis-related proteins. An allergenic protein in peach with a molecular weight of 9 kd was first described by Leonart et al¹² and subsequently identified by Pastorello et al¹³ as an nsLTP, being a major allergen of Prunoideae fruits. Lipid transfer proteins (LTPs) are ubiquitously expressed in plants, and an increasing number of those proteins have already been identified as allergens. Sensitization to nsLTPs is mostly observed in Mediterranean areas, and sensitization to this protein family is frequently associated with systemic reactions.¹⁴⁻¹⁷ Because nsLTPs have been described as panallergens in many foods, especially those of the Rosaceae family (eg, peach, cherry, and apple), hazelnut, tomato, maize, grape, lettuce, citrus fruits, and asparagus (International Union of Immunological Societies Allergen Nomenclature Subcommittee, <http://www.allergen.org>), concomitant food allergies caused by cross-reactivity of IgE antibodies have been observed. Therefore avoidance of a broad spectrum of fruits and vegetables is sometimes required, resulting in a reduced quality of life and even nutritional deficiencies.

The aims of the present study were to investigate the reduction of allergenicity of transgenic fruits showing a suppressed allergen expression *in vivo* by means of SPTs and to investigate the heritability of this effect on the next generation of plants. In addition, results of SPTs were confirmed by using *in vitro* mediator release assays. Our data show a remarkable reduction of allergenicity in the majority of tested patients and stability of the inhibition of allergen expression in first-generation (T0) and second-generation (T1) transgenic fruits.

METHODS

Patients and sera

Inclusion criteria for the selection of patients were a clear history of tomato allergy identified by means of immediate reactions after ingestion of tomato, a positive SPT response with tomato extract, specific IgE to tomato measured with the CAP FEIA (f25; Phadia, Uppsala, Sweden), and IgE binding to natural (n) Lyc e 3. Oral challenge tests were not performed because of frequently severe reactions reported from nsLTP-sensitized patients. All patients were recruited in Castellón, Spain. Only adults with a mean age of 27.6 years (range, 22-41 years) were included in the study. Sera from nonallergic subjects and allergic patients without tomato allergy and without specific IgE to tomato were taken as negative controls. Patients provided written informed consent. The study was approved by the Ethics Committee of the General Hospital from Castellón, Spain.

SPTs

SPTs were performed according to standard procedure as shown in the European Academy of Allergy and Clinical Immunology

position paper¹⁸ by using commercial tomato extract (ALK-Abelló, Madrid, Spain) and by using the prick-to-prick method¹⁹ with the cultivar microtom. Prick-to-prick tests were performed in duplicate or triplicate on the volar surface of the forearm with wild-type (WT) and transgenic tomatoes. Histamine dihydrochloride, 10 mg/mL, and PBS (pH 7.4) solution served as positive and negative controls, respectively. The SPT response was considered positive when the wheal diameter was larger than 3 mm after 15 minutes.

Statistical analysis

Differences in allergenic potencies of extracts of WT versus transgenic plants, as determined by using SPTs, were tested for statistical significance by using the Student *t* test for paired samples. A *P* value of less than .05 was considered a significant difference between 2 groups.

Generation of transgenic tomato plants

The first generation of transgenic tomato plants, T0, showing suppressed Lyc e 3 expression, was developed as previously described.⁵ Thirty seeds of each transgenic line of T0 plants and WT seeds were surface sterilized to test the heritability of the transgenic effect as follows: 3 minutes in 70% ethanol and 10 minutes in 1% HClO, followed by 3 times washing with distilled water. Sterilized seeds were germinated with 16-hour light/8-hour dark intervals on Murashige Skoog media containing 2% (wt/vol) sucrose, 0.1% (wt/vol) morpholinoethane sulfonic acid (pH 6.1), and 50 mg/L kanamycin. After 2 to 3 weeks, kanamycin-resistant plants were transferred to the greenhouse and used for further analysis. Plants were cultivated in soil with 16 hours of supplemental light followed by 8 hours of darkness. Temperature was chosen to be 25°C/20°C according to the day/night cycles.

Preparation of protein extracts from tomato fruits

Preparation of peel and pulp extracts from commercial tomatoes was performed by using the low-temperature method, as described elsewhere.⁵ Tomatoes (cultivar microtom) were either separated into fractions of peel and pulp, or protein extracts from whole fruits were used. For histamine release assays, whole fruits were homogenized in 1 mL of PBS (150 mM NaCl, 2 mM KCl, and 6 mM Na₂HPO₄, pH 7.1) per gram of tomato material. Extraction for 3 hours at 4°C was followed by centrifugation and collection of supernatant. Protein content was determined according to the method of Bradford.²⁰ For practical reasons (amount of material available), peel of cultivar microtom fruits was ground in liquid nitrogen, incubated in 4× SDS-containing running buffer, and centrifuged, and the supernatant was directly applied to SDS-PAGE.

SDS-PAGE and immunoblotting

Allergen extracts (20 μ g of total protein/cm) were separated by SDS-PAGE according to the method of Laemmli²¹ under nonreducing conditions in a Mini-Protein 3 cell (Bio-Rad, Munich, Germany). Proteins were transferred to 0.2- μ m nitrocellulose membranes (Hybond C; Amersham Pharmacia Biotech, Braunschweig, Germany or Schleicher & Schuell, Dassel, Germany) by semidry blotting for 1 hour at 0.8 mA/cm² (Phase, Lübeck, Germany). Membranes were blocked with 5% skimmed milk/TBST (20 mM Tris, 500 mM NaCl, 0.05% (v/v) Tween 20, pH 7.4) for 2 hours, cut into strips, and incubated with 1:10 diluted patients' sera overnight. Immunostaining of bound IgE was performed with a mouse anti-human IgE alkaline phosphatase (AP)-labeled antibody (PharMingen, San Diego, Calif) diluted 1:750 and incubated for 4 hours. IgG binding of a rabbit serum raised against ragweed profilin (1:10,000) and against hazelnut nsLTP, Cor a 8 (1:10,000), respectively, was detected with a goat

TABLE I. Clinical characteristics of patients with tomato allergy

Patient ID	Age (y)	Sex	Symptoms after ingestion of tomato	SPT (mm mean diameter)	CAP tomato, kU/L (CAP class)	Allergic symptoms to other foods
CS-001	41	F	OAS, SR	14	51.5 (4)	Hazelnut, peanut, soy, lentils, peas, corn
CS-003	28	F	OAS, SR, R, G	11	15.8 (3)	Peach, cherry, nectarine, apple, pear, kiwi, walnut, peanut, corn
CS-005	22	M	OAS, G	8	1.6 (2)	Peach, hazelnut, almond, walnut, peanut, peas
CS-010	22	F	OAS, SR	5	9.1 (3)	Peach, apricot, nectarine, corn
CS-011	25	M	OAS, SR, R, G, CV	8	5.0 (3)	Peach, cherry, apricot, nectarine, apple, pear, kiwi, hazelnut, almond, walnut, corn

F, Female; M, male; OAS, oral allergy syndrome; SR, skin reactions; R, respiratory tract; G, gastrointestinal tract; CV, cardiovascular system.

anti-rabbit serum that was biotin conjugated (1:6000) and streptavidin coupled with AP. Visualization was carried out by using enhanced chemiluminescence with CDP Star (Tropix, Bedford, Mass) or according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Basophil histamine release and histamine ELISA

The basophil histamine release assay was performed with resensitized basophils from a nonallergic donor, as previously described.¹⁰ For stimulation of the cells, allergens were diluted to a final concentration of 10, 100, 1000, and 10,000 ng/mL. Released histamine was measured by using a commercial competitive enzyme immunoassay (ImmunoTech, Marseille, France). After subtraction of the spontaneous release, the allergen-induced histamine release was calculated as a percentage of the total amount of histamine determined after lysis of the basophils by means of 2-fold freezing and thawing. Tomatoes were dialyzed overnight (4°C) toward PBS buffer in a slide-A-lyzer dialysis cassette (Pierce, Cheshire, United Kingdom) to reduce the endogenous content of histamine. Determination of the content of endogenous histamine in tomato fruits was performed as described above. Nondialyzed tomato extracts were diluted 1:90, 1:900, 1:1800, and 1:9000, and dialyzed extracts were diluted 1:2, 1:6, 1:18, and 1:54. The extracts were acylated for 2 hours, washed, and incubated with histamine conjugate-AP for 2 hours. After a washing step, bound enzymatic activity was detected by means of addition of a chromogenic substrate, according to the instructions of the manufacturer.

RESULTS

Characterization of patients with tomato allergy

Five Spanish patients with immediate symptoms after ingestion of tomato and IgE sensitization to Lyc e 3 were included. Three patients were female, and 2 were male. The most common symptom was the oral allergy syndrome, followed by skin reactions and gastrointestinal and respiratory problems. One patient experienced a decrease of blood pressure. Clinical characteristics are summarized in Table I. SPTs with commercial tomato extract resulted in mean diameters ranging from 5 to 14 mm, being defined as positive. Specific IgE titers to tomato extract ranged from 1.6 to 51.5 kU/L (Table I). Western blot experiments with tomato peel extract on the solid phase showed strong IgE binding to nLyc e 3 for all 5 sera. Additionally, all sera recognized several other proteins at about 14, 21, 33, and/or 55 kd in extracts from tomato peel and pulp (Fig 1).

In addition to tomato, patients had symptoms after ingestion of peach and corn (n = 4), followed by hazelnut, walnut, peanut, and nectarine (n = 3, Table I). SPTs with other extracts (eg, latex and pollen extracts, such as timothy grass, birch, hazel, plane, mugwort, *Parietaria judaica*, and olive) were performed. All patients had positive SPT responses to mugwort pollen, 3 patients had positive SPT responses to plane, and 2 patients had positive SPT responses to timothy grass pollen. Moreover, 1 patient had skin reactions to latex, olive, and birch pollen (data not shown).

Generation of transgenic tomato fruits and T1 generation thereof

The *NptII* gene coding for kanamycin resistance has been included in the transformation vector to allow selection of transgenic plants. Heritability of the transgene effect was analyzed in T1 plants. Thirty seeds of each T0 transgenic line were germinated on selective medium, and kanamycin-resistant shoots were selected for further analysis. According to Mendel's law, a ratio of 3:1 (sensitive to resistant shoots) should be obtained in case the transgene has integrated into a single locus of the genome. Three T0 plant lines (line 8, 23, and 36) showed a 3:1 segregation, whereas 2 other plant lines (line 11 and 31) showed a 1:1 segregation, indicating multiple insertions of the transgene. Plant lines 8, 23, and 36 were selected for further experiments to allow selection of homozygous plants. Ten kanamycin-resistant seedlings (T1 plants) were transferred into the greenhouse and used for the subsequent analysis to test heritability of RNAi-mediated gene silencing. Silencing of Lyc e 3 in tomato fruit extracts was verified by means of immunoblotting with a Cor a 8-specific rabbit serum, which cross-reacted strongly with Lyc e 3 in protein extracts from WT fruits but not with protein extracts from all 5 transgenic plant lines (Fig 2). These data suggest that the RNAi-mediated gene silencing was stably inherited by the next generation.

Reduced allergenic potency of transgenic tomatoes determined *in vitro* by histamine release assays

In vitro basophil histamine release assays were performed with the T0 plants (line 8) and the corresponding T1 plants (line 8.26) to investigate whether the

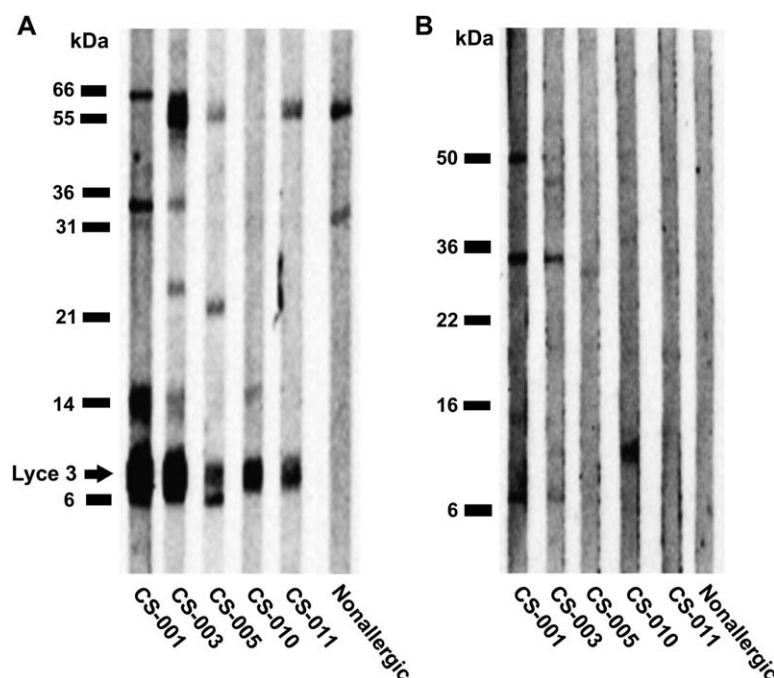


FIG 1. Differences in reactivity of sera from patients with tomato allergy to tomato extracts of peel versus pulp. Results of SDS-PAGE and IgE immunoblotting with sera of patients with tomato allergy (CS-001 to CS-011) and tomato peel (**A**) or pulp (**B**) extract on the solid phase are shown. Serum from a nonallergic subject was used as a negative control. IgE binding to Lyc e 3 is indicated by an arrow.

RNAi-mediated allergen suppression leads to decreased allergenic potency. For T0 and T1 lines, no Lyc e 3 expression could be detected by means of Western blotting (Fig 2). Stripped basophils from a nonallergic donor were passively sensitized with sera from patients with tomato allergy and subsequently incubated with tomato extracts. Tomatoes are known to contain high amounts of endogenous histamine, and therefore the extracts were dialyzed extensively. Removal of histamine was measured after dialysis with histamine ELISA (data not shown). The results of the basophil histamine release assays are shown for 3 patients' sera (Fig 3). Extracts from both transgenic and WT lines induced a significant release of histamine from the basophils. There was no apparent difference between the WT and transgenic lines for serum CS-001 when comparing the highest amount of released histamine (approximately 60%). A maximum of approximately 40% and 50% of released histamine with extracts from the WT line was determined for sera CS-003 and CS-010, respectively. The mediator release in response to extracts from transgenic lines was approximately 20% for CS-003 and approximately 40% for CS-010 when using the highest concentration of extracts. Moreover, 10- to 100-fold higher amounts of extracts of transgenic fruits were required to induce the same amount of histamine release compared with extracts from WT fruits. Half maximal histamine release was calculated: 0.01 $\mu\text{g/mL}$ (CS-001), 0.4 $\mu\text{g/mL}$ (CS-003), and 0.3 $\mu\text{g/mL}$ (CS-010) for extracts from WT plants and 0.4 to 0.6 $\mu\text{g/mL}$ (CS-001), 8.5 to 10 $\mu\text{g/mL}$ (CS-003), and 6 to 8 $\mu\text{g/mL}$ (CS-010) for extracts from transgenic plants. There were no

differences detectable between the T0 and T1 lines in regard to allergenic potencies. For each serum, both curves are almost identical, indicating stable allergen suppression in both generations (Fig 3). For sera CS-005 and CS-010, the basophil histamine release test was also performed and tended to show the same results as for the other sera. However, because of the low tomato-specific IgE titers, no significant mediator release could be determined.

SPTs reveal a reduced allergenic potency of transgenic tomatoes

Mean skin reactivity (out of triplicates) to WT plants (cultivar microtom) varied from 6- to 12.5-mm wheal diameter, whereas mean skin reactivity to transgenic plants (cultivar microtom) varied from 2- to 10-mm wheal diameter (Fig 4). Similar results of skin reactivity were obtained by using prick-to-prick tests with commercial tomatoes in comparison with WT fruits (cultivar microtom). For 3 of 5 patients (sera CS-003, CS-005, and CS-011), mean skin reactivities to transgenic fruits were less than 3 mm and therefore judged as negative. In those 3 patients residual skin reactions to the transgenic plants were less than one third of the reactivity to WT plants (CS-003, 19%; CS-005, 30%; CS-011, 29%), whereas a higher residual reactivity was observed in the 2 other patients (CS-001, 80%; CS-010, 47%). Histamine dihydrochloride as a positive control led to wheal diameters ranging from 7 to 10 mm.

For statistical analysis of supposed differences of wheal diameters obtained by WT and transgenic plants, the differences of 5 mean values from each group were compared pairwise. Differences in skin reactivity between WT and

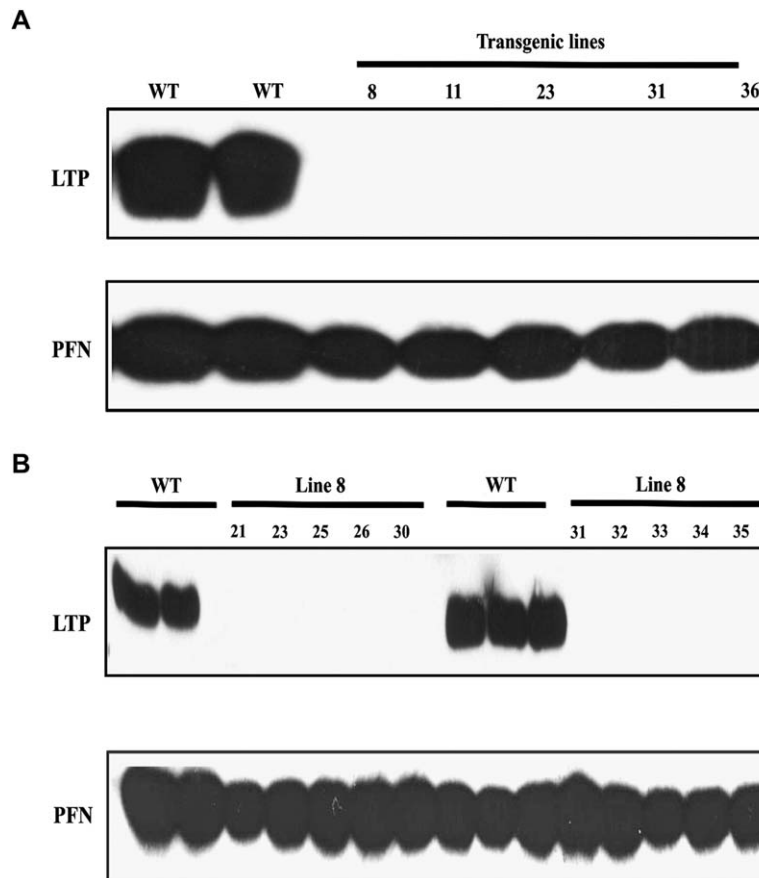


FIG 2. Strong suppression of Lyc e 3 accumulation in transgenic tomato fruits by means of RNAi-mediated gene silencing. IgG immunoblotting with transgenic lines T0 (A) and T1 (B) and untransformed plants (WT). T1 transgenic plants represent 10 different lines that are independent offspring derived from line 8. As a loading control, IgG binding to profilin was detected by using a rabbit anti-ragweed profilin serum. PFN, Profilin.

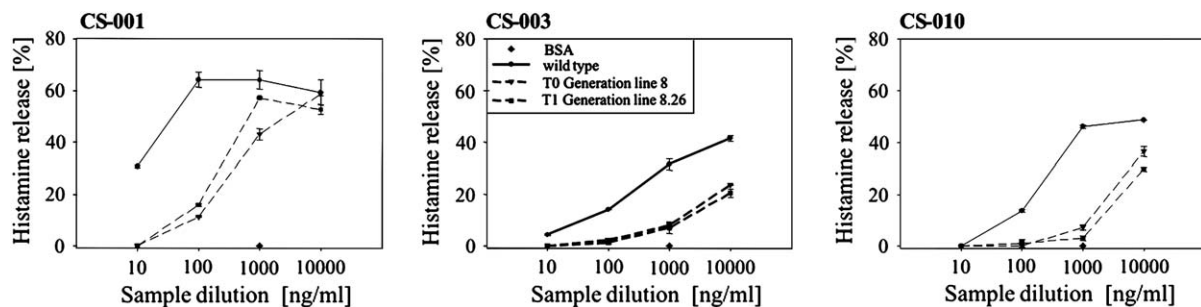


FIG 3. Histamine release tests reveal a reduced allergenic potency of allergen-suppressed transgenic tomatoes *in vitro*. Passively sensitized basophils were stimulated with increasing doses of protein extracts from WT and transgenic fruits from T0 and T1 plants. BSA was used as negative control. Means and error bars of a double determination are shown.

allergen-suppressed fruits were significant ($P = .0053$, Student t test for paired samples).

Gene silencing induces no upregulation of additional IgE-binding proteins

IgE immunoblots with peel extracts of microtom WT and transgenic fruits were performed. Serum CS-001 was

used to assess whether upregulation of other proteins had led to the weak changes of allergenic potency of transgenic fruits indicated by SPTs in this patient. No IgE binding to Lyc e 3 could be detected in transgenic fruits compared with that seen in the WT fruits. Furthermore, no upregulated expression of additional IgE-reactive tomato proteins was observed by using this serum (Fig 5, A).

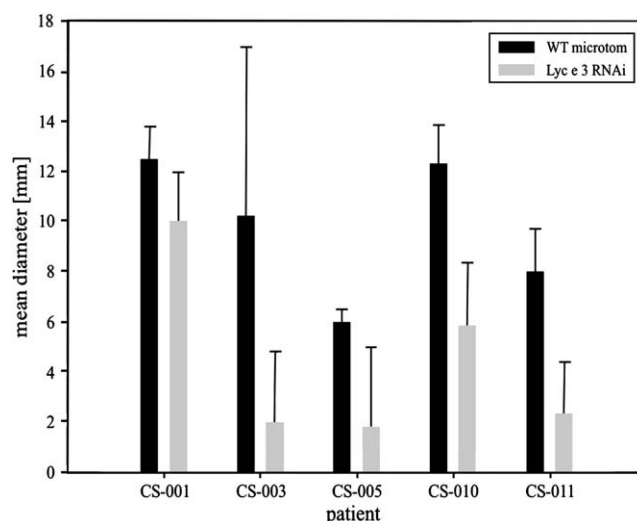


FIG 4. SPTs with Lyc e 3–silenced tomatoes indicate a significant reduction of skin reactivity in comparison with WT fruits. Prick-to-prick tests were performed in 5 patients with tomato allergy sensitized to Lyc e 3 with WT and transgenic tomato fruits of T1 plants. Means and error bars of wheal diameters of triplicates are shown.

Serum of a nonallergic subject showed no reactivity, suggesting that no unspecific binding to Lyc e 3 in WT plants occurred (Fig 5, B). Differences in the amount of protein blotted could be excluded because of similar expression of profilin (Fig 5, C).

DISCUSSION

In this study we demonstrated for the first time using *in vivo* tests that RNAi is a suitable method to reduce the allergenic potency of tomato fruits from the parental and offspring plants.

The allergenic potency of transgenic tomato fruits was investigated by using the *in vitro* basophil histamine release assay. We obtained the same reduction of allergenic potency within both the T0 and T1 plants compared with WT plants, confirming the results obtained by using immunoblotting with the specific rabbit serum. In addition, SPTs showed a significantly reduced or almost abolished skin reaction to transgenic fruits in 4 of 5 patients. In fact, 3 of 5 patients had negative results (<3 mm) on prick-to-prick tests. Both the *in vitro* and *in vivo* assays generally revealed a low residual allergenic potency of the transgenic plants. These results are in agreement with those of Gilissen et al,³ who have shown a strong but incomplete reduction of skin reactivity in response to leaves from Mal d 1–silenced apple trees. This phenomenon could possibly be explained by residual LTP accumulation in transgenic plant lines, although the residual expression could not be detected by means of immunoblotting. A competitive ELISA with a rabbit antiserum raised against LTP from hazelnut was performed to quantify the suppression of Lyc e 3 directly. Considering the fresh weight, the concentration of LTP was approximately 0.4% for WT extract

and 0.02% for transgenic line 8.26 (data not shown in detail), confirming that the silencing effect was strong but still incomplete.

Moreover, additional tomato proteins that were not targeted by gene silencing might have contributed to the residual allergenicity of the transgenic lines. Although all patients were mainly sensitized to Lyc e 3, they also showed IgE binding to other proteins in extracts of tomato peel and pulp (Fig 1), and additional allergens with conformational epitopes might have been undetectable because of the denaturing conditions of SDS-PAGE. Therefore simultaneous silencing of multiple allergens will be required to further reduce the allergenicity of tomatoes.

Here we showed that the transgenic fruits had a reduced allergenic potency *in vivo*. Whether a genetically modified tomato fruit is still capable of causing allergic symptoms after ingestion has to be assessed in oral challenges. Such experiments have thus far not been performed with transgenic fruits. A double-blind, placebo-controlled food challenge with a hypoallergenic mutant of the apple allergen Mal d 1 was performed by Bolhaar et al²² and did not result in allergic reactions after ingestion.

In our study 4 patients presented dramatically reduced skin reactivity to Lyc e 3–silenced tomato fruits. However, the reduction of skin reactivity to the transgenic fruits was less impressive (20% reduction compared with the WT) in one patient (CS-001). This could be due to an IgE reaction of this patient to other tomato proteins, especially if those proteins were upregulated as a result of the genetic modification. Immunoblots were prepared from the same fruits that were used for SPTs and probed with serum from the discrepant patient to verify this hypothesis. No additional IgE-reactive protein was observed in the transgenic plants, which was also indicated by results of the *in vitro* histamine release assay, showing a similar maximal

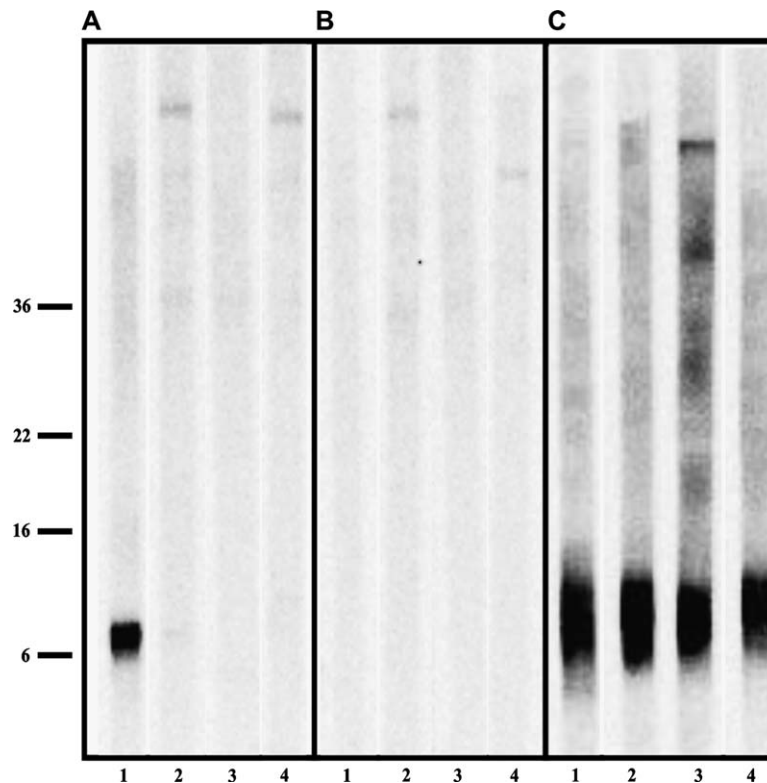


FIG 5. RNAi-mediated gene silencing almost completely reduced accumulation of Lyc e 3 in tomato peel. IgE immunoblotting was performed with peel extract of WT (lane 1) and transgenic (lanes 2-4) fruits of T1 plants on the solid phase. Membranes were probed with serum from patient CS-001 (A) and a nonallergic subject (B). Rabbit anti-profilin (C) was used as a loading control.

histamine release for both WT and transgenic fruits. Those findings are in accordance with results of Herman et al,² who were unable to detect any changes in protein pattern after silencing the soy protein Gly m Bd 30K. Moreover, we can exclude that the partially retained skin reaction observed in this patient might be caused by a hyperreactivity to the high content of endogenous histamine in tomato (2.2 mg/100 g; Allergie-Centrum Charité, <http://www.allergie-centrum-charite.de/fileadmin/documents/histaminintoleranz.pdf>) because the histamine dihydrochloride control led to comparable reactions in all 5 patients. In addition, quantification of histamine content revealed no apparent differences in the amount of endogenous histamine in the transgenic fruits (350-2100 µg/100 g tomato) in comparison with the WT fruits (700-1700 µg/100 g tomato, data not shown). Interestingly, this patient had the highest concentration of specific IgE to tomato, which was 3.3- to 32.2-fold higher than in the remaining 4 patients. The missing concordance between SPTs and the histamine release assay might result from a discrepancy between specific IgE in serum, which was used for passive sensitization of basophils, and IgE bound to mast cells in the skin, which might represent a different antigen recognition profile in this patient. This hypothesis is supported by observations of Kubo et al,²³ who found that the level of cell-bound IgE is stabilized and that mast cells retain

the capability of maintaining a long-term, antigen-specific allergic response, whereas serum IgE levels decrease rapidly.

Reduction of allergen expression in the first generation (T0) of Lyc e 3-silenced tomato plants was described in our former study.⁵ In the present study the heritability and stability of silencing was verified in the next generation by means of immunoblotting and functional assays. Preliminary results indicate that the suppression is also stable in T2 plants. No IgE binding to LTP was detected for the second generation, confirming the suppression is stable. Inhibition of Lyc e 3 expression was achieved by means of RNAi in transgenic tomato plants. Lyc e 3 belongs to the group of nsLTPs, the precise function of which is not well understood in plants yet. It is currently discussed whether nsLTPs are involved in formation of the cutin layer and play a role in plant defense. However, downregulation of Lyc e 3 did not cause any phenotypic alterations.

Thus T0 and T1 Lyc e 3-deficient tomato plants were indistinguishable from WT plants. They flowered at the same time, and fruits were unaltered in size and numbers per plant. This could be explained by the observation that nsLTPs are encoded by large gene families. There are more than 30 putative nsLTP genes in the genome *Arabidopsis thaliana*,^{24,25} and other members of this gene family might be functionally equivalent and therefore

replace the function or functions of silenced Lyc e 3. The commercial and practical feasibility strongly depends on the acceptance of transgenic food by the population. Although gene silencing of allergens might provide a benefit for patients, the problem of cross-reactivity to foods containing homologous allergens will still exist.

In summary, our study on transgenic tomato plants provided a proof of principle in the area of allergen suppression. RNAi technology was found to be an effective tool to generate foods with reduced allergenicity *in vivo*. Moreover, suppression of allergen expression was stable in the next generation plants. Fruits with reduced allergen expression might, in the future, be promising as an alternative to food avoidance and might lead to an improvement of the quality of life of patients with food allergy.

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