

Reduced allergenicity of tomato fruits harvested from *Lyc e 1*-silenced transgenic tomato plants

Lien Quynh Le, MSc,^a Vera Mahler, MD,^b Yvonne Lorenz, MSc,^c Stephan Scheurer, PhD,^c Sophia Biemelt, PhD,^a Stefan Vieths, PhD,^c and Uwe Sonnewald, PhD^a

Erlangen and Langen, Germany

Background: Profilin is a small actin-binding protein that contributes to the allergenic potency of many fruits and vegetables, including tomato. Two highly similar genes encoding tomato profilin have been isolated and designated as allergen *Lyc e 1.01* and *Lyc e 1.02*.

Objective: The aim of the study was to generate profilin-reduced hypoallergenic tomato fruits by silencing of both genes in transgenic tomato plants by means of RNA interference (RNAi).

Methods: The efficiency of gene silencing was documented by means of Northern blotting, immunoblotting, and skin prick testing.

Results: Quantification of the remaining protein revealed that profilin accumulation in transgenic fruits was decreased 10-fold compared with that seen in untransformed controls. This decrease was sufficient to cause a reduced allergenic reactivity in patients with tomato allergy, as determined with skin prick tests. Because most patients with tomato allergy are not monosensitized to profilin, the IgE reactivity to the profilin-silenced tomato fruits *in vivo* varied widely between individuals tested.

Conclusion: We could demonstrate the efficient silencing of both profilin genes in transgenic tomato plants using RNAi. This resulted in *Lyc e 1*-diminished tomato fruits, providing proof of concept and demonstrating that RNAi can be used to design allergen-reduced food. However, simultaneous silencing of multiple allergens will be required to design hypoallergenic tomatoes.

Clinical implications: Our findings demonstrate the feasibility of creating low-allergenic food by using RNAi. This concept

constitutes a novel approach to allergen avoidance. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: *Lyc e 1*, RNA interference, tomato allergy, allergen avoidance

Profilin is an ubiquitous protein found in all eukaryotic cells, where it acts as an actin- and phosphatidylinositol 4,5-bisphosphate-binding molecule involved in the regulation of the cytoskeleton and signal transduction.

Plant profilins have been described as panallergens in several food sources and pollen. Sensitization to profilins is observed in approximately 20% of patients with pollen allergy, and IgE to pollen profilin is strongly cross-reactive to profilins of fruits and vegetables.¹ Whereas the IgE binding to profilins in food sources containing Bet v 1-homologous proteins shows similar frequencies as in pollen, the IgE sensitization to profilins is more prominent in exotic fruits, reaching up to 70% in banana or pineapple.² Recently, *Lyc e 1* was identified as a minor allergen in tomato (*Lycopersicon esculentum*) fruits, showing IgE reactivity in 22% to 26% of patients with tomato allergy.^{3,4} In these patients the IgE reactivity to tomato profilin comprises up to 42% of the overall IgE reactivity to tomato fruit extracts. Residual IgE reactivity is most likely due to other tomato allergens, such as *Lyc e 2* (β -fructofuranosidase), *Lyc e 3* (nonspecific lipid transfer protein [LTP]), polygalacturonase, pectinesterase, chitinase, superoxide dismutase, pathogenesis-related protein 10, and thus far unknown allergens.⁵⁻¹⁰ In patients with tomato allergy and multiple sensitizations to other food sources and pollen, IgE directed to *Lyc e 1* showed a strong cross-reactivity with profilin from birch pollen, Bet v 2, and a weaker but significant cross-reactivity with profilins from celery, carrot, cherry, and pineapple.³ IgE directed against tomato profilin can be responsible for cross-reactivity between unrelated plant sources, but IgE epitope differences within the profilin family exist.¹¹ Moreover, *Lyc e 1* showed allergenic potency through its capability to trigger release of inflammatory mediators from human basophils *in vitro*.³ Therefore it is likely that *Lyc e 1* contributes substantially to clinical symptoms in patients with tomato allergy.

From ^athe Department of Biochemistry, Friedrich-Alexander University Erlangen-Nuremberg, Erlangen; ^bthe Department of Dermatology, Friedrich-Alexander University Erlangen-Nuremberg, University Hospital, Erlangen; and ^cPaul-Ehrlich-Institut, Langen.

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Reprint requests: Uwe Sonnewald, PhD, Friedrich-Alexander Universität Erlangen-Nürnberg, Lehrstuhl für Biochemie, Staudtstr. 5, 91058 Erlangen, Germany. E-mail: usonne@biologie.uni-erlangen.de. 0091-6749/\$32.00

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Abbreviations used

LTP: Lipid transfer protein
 RNAi: RNA interference
 SPT: Skin prick test
 WT: Wild-type

Plant profilin is encoded by a small gene family. In *Arabidopsis thaliana* 5 profilin-encoding genes (*PRF1* to *PRF5*) with distinct but overlapping expression patterns have been described.¹² Downregulation of *PRF1* expression by antisense inhibition or insertional mutagenesis impaired development of seedlings and cell elongation.^{13,14} Mature plants showed a dwarf phenotype and early flowering. Thus individual members of the profilin gene family play essential roles during plant development; however, distinct isoforms have overlapping functions and are able to partially substitute for each other.

Different gene-silencing strategies have been applied to decrease the accumulation of allergenic proteins in soybean, rice, apple, and tomato to create low-allergenic food sources.^{6,15-17} Gene silencing (by means of RNA interference [RNAi] technology) offers the possibility to inhibit expression of entire gene families and to restrict the genetic manipulation to target tissues only. Thereby the risk of unwanted side effects is minimized. Simultaneous inhibition of gene family members by using RNAi can be achieved by selecting nucleotide regions of high homology often found within coding regions. Selecting less conserved 5'- or 3'-untranslated regions allows inhibition of individual members of gene families. Although systemic silencing has been shown for nonplant target genes (ie, viruses), silencing of endogenous genes seems to be cell autonomous. Thus by selecting appropriate promoter sequences, fruit-, seed-, or pollen-specific silencing of target genes can be envisaged. By following conventional breeding methods, tissue-specific silencing of gene families cannot easily be achieved.

Here we report on the successful inhibition of *Lyc e 1* expression in transgenic tomato plants. Inhibition was achieved by using constitutive expression of *Lyc e 1*-specific hairpin RNAs. On the basis of quantitative ELISA assays, profilin accumulation in transgenic tomato fruits was 10-fold less than in untransformed controls. When challenged with transgenic fruit extracts, patients with tomato allergy had strongly reduced skin prick test (SPT) reactivity, indicating a reduced allergenic reactivity *in vivo* caused by *Lyc e 1* suppression.

METHODS

Selection and characterization of patients with tomato allergy

Patients with tomato allergy were included on the basis of case history (oral allergy syndrome, gastrointestinal symptoms, or contact urticaria syndrome, respectively, on contact or ingestion of tomato fruit), positive prick-to-prick test results with tomato fruit, or *in vitro*

basophil histamine release. Tomato-specific serum IgE was demonstrated by using CAP FEIA or IgE immunoblotting. Age and clinical characterization of patients are summarized in Table I. Approval of the local ethics committee (Faculty of Medicine, University of Erlangen-Nuremberg, no. 3344) for this study and written informed consent of all participants were obtained. Consecutive patients with a convincing history of tomato allergy and tomato-specific serum IgE were screened *in vitro* (CAP FEIA or IgE immunoblotting) for sensitization to profilin (Table I). As control specimens, sera from individuals with tomato allergy without sensitization to profilin and from nonallergic individuals were included (data not shown).

Plant material and growing condition

Transgenic (LTP- and profilin-reduced) and untransformed control tomato plants (*Lycopersicon esculentum*, cultivar MicroTom) were grown in tissue culture under 16 hours light/8 hours dark intervals on Murashige Skoog medium containing 2% (wt/vol) sucrose. After initial characterization of *in vitro* grown plants, tomato plants were transferred to the greenhouse. Plants were cultivated in soil with 16 hours of supplemental light followed by 8 hours of darkness. The temperature regimen followed a day/night cycle of 25°C/20°C.

Preparation of tomato protein extracts

For ELISA inhibition assays, whole red tomato fruits were homogenized with a biohomogenizer in PBS (pH 7.1, 150 mmol/L); 1 mL of PBS was used per 1 g of tomato. Proteins were extracted for 3 hours at 4°C under gentle agitation. After centrifugation at 15,180g, the supernatant was collected and filtered (0.2 µm; Schleicher & Schuell, Dassel, Germany). The extracts were lyophilized and stored at -80°C.

For Western blotting, fruit or leaf samples (approximately 100 mg fresh weight) were homogenized in extraction buffer containing 50 mmol/L Tris-HCl (pH 6.8), 5 mmol/L MgCl₂, 1 mmol/L EDTA, 1 mmol/L ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid, 5 mmol/L β-mercaptoethanol, 0.1 mmol/L Pefabloc proteinase inhibitor, and 15% glycerol. Samples were centrifuged for 5 minutes at 4°C, and protein content of the supernatant was determined according to the method of Bradford¹⁸ with the BioRad protein assay solution (BioRad Laboratories, Hercules, Calif).

ELISA inhibition

Ninety-six-well Maxisorb plates (Nunc, Wiesbaden, Germany) were coated with 0.5 µg of rLyc e 1³ diluted in PBS overnight at 4°C. The plates were blocked with PBS plus 0.05% Tween 20 plus 1% BSA for 1 hour. After washing 3 times with PBS plus 0.05% Tween 20, each well was incubated with 50 µL of diluted human serum (PEI 131; 1:10 with PBS) as well as with 50 µL of tomato extracts (0.02, 0.2, 2, 20, and 200 µg/mL total protein) for 1 hour. As a positive control, rLyc e 1 (20 µg/mL) was applied as an inhibitor. Subsequently, plates were washed and incubated with a monoclonal anti-human IgE horseradish peroxidase (Southern Biotechnology Associates Inc, Birmingham, Ala) antibody for 1.5 hours. After 3 additional washing cycles, visualization was performed with tetramethylbenzidine and hydrogen peroxide for 0.5 hours. The color reaction was stopped with 3M H₂SO₄, and absorption was measured at 450 nm. Duplicate determinations were performed in each case. Inhibition rates were calculated as the percentage of noninhibitor values of the sera under consideration of the nonspecific binding control (no serum and antibody).

Western blot analysis

Ten micrograms of total soluble protein extracts obtained from fruits of the different tomato genotypes or 50 µg of total soluble leaf

TABLE I. Clinical and serologic characterization of patients with tomato allergy

Patient identifier	Profession	Age (y)	Clinical symptoms on contact or ingestion of tomato	CAP FEIA tomato	CAP FEIA bromelain	CAP FEIA profilin (rBet v 2)
002	Medical doctor	37	Oral allergy syndrome	3.08 KU/I (class 2)	1.61 kU/L (class 2)	25.7 kU/L (class 4)
013	Master for nutrition and ecotrophology	28	Oral allergy syndrome (uncooked tomato)	Positive IgE immunoblot result	<0.35 kU/L (class 0)	0.36 kU/L (class 1)
023	Businesswoman	38	Oral allergy syndrome	0.52 kU/L (class 1)	0.39 kU/L (class 1)	Positive IgE immunoblot result
PEI 131	Housewife	55	Oral allergy syndrome	11.0 kU/L (class 3)	<0.35 kU/L (class 0)	12.6 kU/L (class 3)

protein was mixed with SDS-containing sample buffer,¹⁹ heat denatured, and separated on 15% (vol/vol) SDS-containing polyacrylamide gels. Subsequently, proteins were blotted onto 0.2- μ m nitrocellulose membranes (Hybond C; Amersham Pharmacia Biotech, Braunschweig, Germany), blocked for 2 hours in 5% skimmed milk/TBST (20 mmol/L Tris, 500 mmol/L NaCl, and 0.1% [vol/vol] Tween 20), and incubated overnight at 4°C with rabbit antiserum raised against ragweed profilin (diluted 1:10000) or with anti-Cor a 8 (hazelnut LTP) antiserum (diluted 1:10000). Immunodetection was done with the ECL system (Amersham Pharmacia Biotech), according to the manufacturer's instructions.

For analysis of tomato-specific IgE binding from human sera, total protein extracts from tomato fruits were prepared and blotted onto nitrocellulose as described above. All blocking and washing steps were performed in buffer A (50 mmol/L sodium phosphate [pH 7.5], 0.5% Tween-20, 0.5% BSA, and 0.05% NaN_3), as previously described.²⁰ Membranes were incubated with 3 mL of 1:5 in buffer A–diluted sera from patients and control subjects. Bound IgE was detected with iodine 125–labeled anti-human IgE antibodies from mouse/goat (Hycor Biomedical, Kassel, Germany) diluted 1:50 in buffer A. Autoradiographies were exposed to Kodak XOMAT film with intensifying screens (Kodak, Heidelberg, Germany) at –70°C for 8 days.

RNAi vector construction

The Gateway technology (Invitrogen, Karlsruhe, Germany) was used to generate an RNAi construct. To this end, a 300-bp cDNA fragment of Lyc e 1.02 (accession no. AY061819; nt 61–360) was amplified by means of PCR from the pCRII-Topo clone described by Westphal et al³ by using the primers 5'PFN-1.02 (5'-ACTTCT-GCTGCTATCATCGGT-3') and 3'PFN-1.02 (5'-CACCTTCAAC-AATCATATTACAC-3'). PCR conditions were as follows: 94°C for 5 minutes followed by 30 cycles of 94°C for 20 seconds; 55°C for 60 seconds and 72°C for 60 seconds; and a final extension at 72°C for 10 minutes. The resulting PCR product was introduced into pENTR/D (Invitrogen) to create an entry clone containing the attL recombination sites. Subsequently, the lambda reconstruction (LR) recombination reaction was performed according to the instructions of the manual to transfer the fragment into the destination vector pK7GW1WG2(II)²¹ containing the attR attachment sites. Thereby the final construct, designated as pGW_PFN_RNAi, was generated, carrying Lyc e 1 in sense and antisense orientation separated by an intron under control of the constitutive cauliflower mosaic viral 35S promoter.

Plant transformation

The final constructs were transferred to *Agrobacterium tumefaciens* CV58C1, carrying the virulence plasmid pGV2260. Tomato cotyledon explants were transformed and regenerated as previously described.²²

RNA isolation and Northern blot analysis

RNA was isolated from tomato fruits essentially as previously described.²³ Twenty micrograms of total RNA was separated by means of electrophoresis on 1.5% formaldehyde-containing agarose gels and blotted onto nylon membranes (GeneScreen; NEN, Boston, Mass) by means of capillary blotting overnight. The membranes were prehybridized and hybridized at 65°C. Radioactive labeling of cDNA fragments was performed with α -³²P-deoxycytidine triphosphate by using the High prime kit (Roche, Mannheim, Germany). After intensive washing, membranes were exposed to x-ray films (Kodak) for 2 to 3 days at –80°C.

cDNA fragments encoding for Lyc e 1.01 (accession no. AJ417553, nt 1–1396), Lyc e 1.02 (accession no. AY061819; nt 61–360), and 18S rRNA (accession no. X51576) were amplified from tomato cDNA⁶ by mean of PCR. Lyc e 1.01 was amplified with the primers 5'PFN-1.01 (5'-ATGAACACTTGCTTTGTGAG-3') and 3'PFN-1.01 (5'-ATGCCTAGAGACTCTGTCTATAAG-3'), Lyc e 1.02 was amplified as indicated above, and tomato 18S rRNA was amplified by using the primers 18S-rRNA-f (5'-CAGCCGCGG-TAATTCAGCTCCAATAG-3') and 18S-rRNA-r (5'-CATCCT-TGGCAAATGCTTTCGAGTTGTTTCG-3'). PCR products were cloned into pCR2.1 vector (Invitrogen). Fragments used for hybridization were excised by using the *Eco*RI restriction sites of the vector.

SPTs

SPTs were performed according to the method of Dreborg.²⁴ Fresh tomato fruit (MicroTom wild-type [WT] and profilin-reduced transgenic fruits) were used for prick-to-prick tests in patients and control subjects, as well as a commercially available extract from tomato (Alk prick N 654; ALK-Scherax, Hamburg, Germany). Control SPT solutions (Histamine 1+999 and 0.9% sodium chloride) were purchased from Allergopharma (Reinbek, Germany). Readings were done after 20 minutes, and mean wheal diameters were determined.

RESULTS

Generation of Lyc e 1–reduced tomato fruits by means of RNAi

An RNAi strategy was followed to generate tomato fruits with reduced Lyc e 1 expression. In tomato Lyc e 1 is represented by 2 isoforms, Lyc e 1.01 and Lyc e 1.02 (accession nos. AJ417553 and AY061819), which share 88.1% identity at the nucleotide level. This degree of identity is sufficient to allow silencing of both isoforms by using 1 RNAi construct. For RNAi, a 300-bp cDNA fragment of Lyc e 1.02 (position 61nt to 360nt) was inserted in sense and antisense orientation into the plant

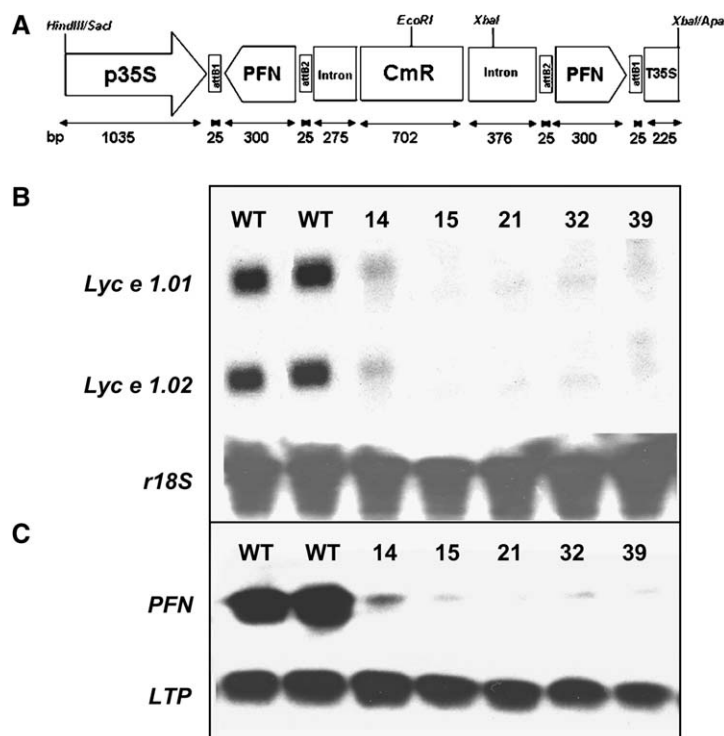


FIG 1. Silencing of *Lyc e 1* expression in transgenic tomato plants. **A**, Schematic drawing of *Lyc e 1* RNAi construct. *p35S*, 35S-CaMV promoter; *T35S*, terminator; *attB1*, *attB2*, recombination sites; *CmR*, chloramphenicol acetyltransferase gene. **B**, Northern blot analysis of transgenic plants. Twenty micrograms of total RNA was loaded on each lane and hybridized with radiolabeled *Lyc e 1.01*, *Lyc e 1.02*, or 18S rRNA cDNA. WT, Untransformed control plants; 14, 15, 21, 32, and 39, 5 independent transgenic lines. **C**, Western blot analysis of transgenic plants. Fruit protein extracts were prepared from 5 different transgenic lines (lines 14, 15, 21, 32, and 39) and untransformed control plants (lines WT). Western blot analysis was performed with profilin- or LTP-specific antisera.

transformation vector pK7GWIWG2(II),²⁴ yielding the final plasmid pGW_PFN_RNAi (for details, see the Methods section). The resulting binary plant transformation vector carries *Lyc e 1* sense and antisense fragments separated by an intron under control of the constitutive 35S cauliflower mosaic viral promoter (Fig 1, A). This binary RNAi construct was transformed into tomato by *A tumefaciens*-mediated gene transfer, and a total of 50 kanamycin-resistant tomato plants were generated and analyzed.

Transgenic plants were screened for loss of *Lyc e 1* RNA and protein in fruit extracts. Protein extracts were separated on SDS-PAGE and blotted onto nitrocellulose membranes. For immunodetection of *Lyc e 1*, rabbit polyclonal antibodies raised against profilin from ragweed were used. As shown in Fig 1, C, the ragweed antibody cross-reacted strongly with *Lyc e 1* in protein extracts from WT fruits. This protein band was faintly detected by the antiserum in fruit extracts from 3 independent transgenic lines (transgenic lines 14, 15, and 32) but not in 2 other transgenic lines (transgenic lines 21 and 39). The same blot was incubated with polyclonal antiserum against LTP to verify that similar amounts of protein had been loaded. As shown in Fig 1, C, the amount of LTP was indistinguishable in protein extracts from WT and transgenic plants.

Total RNA from tomato fruits was isolated and subjected to Northern blot analysis to confirm these results at the mRNA level. Accumulation of *Lyc e 1* mRNA was probed by hybridizing the RNA blot with radiolabeled cDNAs from *Lyc e 1.01* and *Lyc e 1.02*. Within the selected nucleotide sequence, *Lyc e 1.01* and *Lyc e 1.02* are 84.4% identical, which allows distinction of both genes under the hybridization conditions used (data not shown). In WT plants both probes detected substantial amounts of *Lyc e 1* mRNA (Fig 1, B). In fruits of transgenic lines, accumulation of *Lyc e 1.01*- and *Lyc e 1.02*-specific mRNA was strongly decreased (line 14) or less than the detection limit (lines 21 and 39). As a loading control, the same blot was hybridized with radiolabeled cDNA from ribosomal 18S RNA. As shown in Fig 1, B, equal amounts of ribosomal 18S RNA were detectable in WT and transgenic plants, indicating that similar amounts of RNA had been loaded.

ELISA inhibition assays reveal a 10-fold reduction in *Lyc e 1* accumulation in transgenic fruits

Dose-dependent IgE inhibition assays were performed to show the suppression of tomato profilin in transgenic

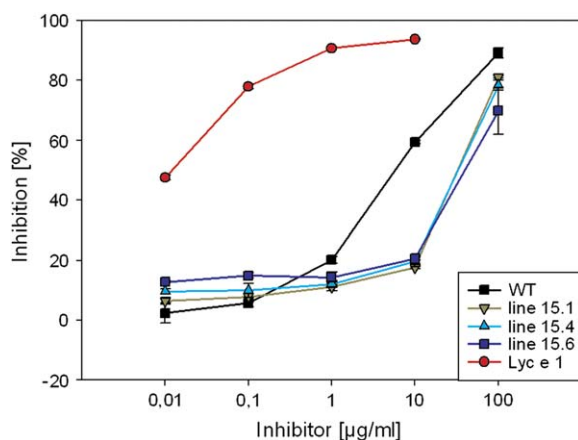


FIG 2. Dose-dependent IgE inhibition was performed with a serum from a patient with tomato allergy (PEI 131) and rLyc e 1 on the solid phase. The patient's serum was incubated with fruit extracts from WT plants and 3 independent extracts of transgenic line 15. Inhibition with rLyc e 1 was used as positive control.

fruits (Fig 2). Microtiter plates were coated with rLyc e 1 and tested for IgE binding of human serum with and without preincubation of inhibitor allergens. After preincubation of serum from a patient with tomato allergy sensitized to tomato profilin with tomato extracts from WT plants (200 µg/mL) or rLyc e 1 protein (20 µg/mL), IgE binding was completely inhibited (Fig 2). Half maximal inhibition values (which reflect the biologic activity of the test extracts) were obtained with approximately 9 µg/mL tomato extract from WT fruits. In contrast, an approximately 10-fold higher protein concentration of extracts from transgenic plants was required to obtain similar inhibition values, indicating a strong suppression of tomato profilin and the potential of reduced allergenicity of fruits after gene silencing (Fig 2).

Constitutive silencing of Lyc e 1 leads to a severe growth retardation of transgenic tomato plants

Remarkably, the transgenic lines exhibited severe growth retardations under greenhouse conditions, emphasizing the essential cellular function of profilin. Although most WT plants reached a final height of 15 to 20 cm and flowered 8 to 10 weeks after transfer to the greenhouse, Lyc e 1–reduced transgenic plants stayed 1.5 to 3.0 times smaller and started flowering not before 12 weeks after germination. A representative picture of 15-week-old WT and transgenic tomato plants is shown in Fig 3, B. Compared with WT plants, fruit and seed set of transgenic plants was decreased, with line 11 forming no fruits at all. The severity of growth phenotype clearly correlated with the degree of gene silencing, as determined by means of immunoblot analysis of profilin accumulation in leaves (Fig 3, A). Leaves were selected for the analysis because some transgenic lines (ie, line 11) remained fruitless.

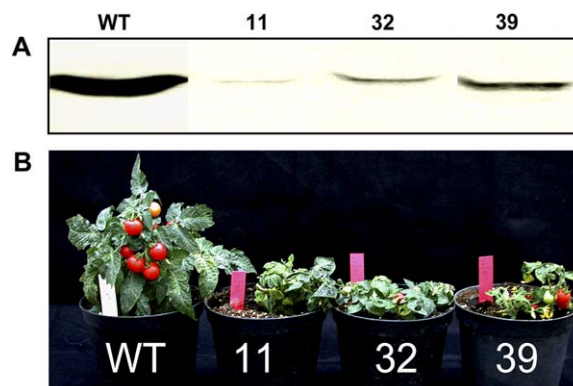


FIG 3. Immunoblot analysis of leaf profilin accumulation (A) and growth phenotype 15 weeks after germination (B) of 3 independent profilin-reduced transgenic tomato and WT MicroTom plants. Western blot analysis was performed with a rabbit anti-ragweed profilin antiserum.

IgE immunoblot analysis with human sera reveals different allergen recognition

Consecutive patients ($n = 16$) with a history of clinically relevant tomato allergy were recruited to test the allergic reactivity to Lyc e 1–reduced tomato fruits *in vitro* and *in vivo*. Specific IgE raised against tomato and rBet v 2 (birch pollen profilin as marker) was detected by using CAP FEIA (Table 1), and subsequently, Lyc e 1–specific IgE was investigated by using IgE immunoblot analysis with blotted fruit extracts from WT, LTP-reduced, and profilin-reduced transgenic plants. This analysis revealed Lyc e 1–specific IgE in 5 patients (data not shown). As controls, sera from 2 nonallergic patients were included in the study (data not shown).

Different allergen recognition patterns were observed in the different patients with tomato allergy. Representative IgE-binding patterns are demonstrated (Fig 4).

Patient 013 was monosensitized to Lyc e 1 and exhibited exclusively specific IgE binding to the 14-kd allergen (Lyc e 1) present in the natural extract of the WT fruits (Fig 4, lane 1), as well as in the LTP-reduced fruit (Fig 4, lane 2). No specific IgE binding was detectable to the extract of the transgenic profilin-reduced tomato fruit (Fig 4, lane 3).

In contrast to patient 013, the sera from patients 023 and 002 contained IgE antibodies binding to tomato allergens of high molecular weight (Fig 4, patients 023 and 002, lanes 1–3) in addition to Lyc e 1–specific IgE antibodies.

In patient 023 there was no specific IgE binding to profilin detectable at 14 kd in the extract from the transgenic profilin-reduced tomato extract (Fig 4, lane 3), whereas the concomitant IgE binding to high-molecular-weight and unrelated tomato allergens remained detectable.

In polysensitized patient 002, a strong IgE binding to Lyc e 1 and several high-molecular-weight allergens was observed (Fig 4, patient 002, lanes 1 and 2). To analyze whether the 60-kd band (visible in Fig 4, serum 002, lane 3) is exclusively present in the profilin-reduced tomato fruit extracts, we analyzed 9 independent fruit

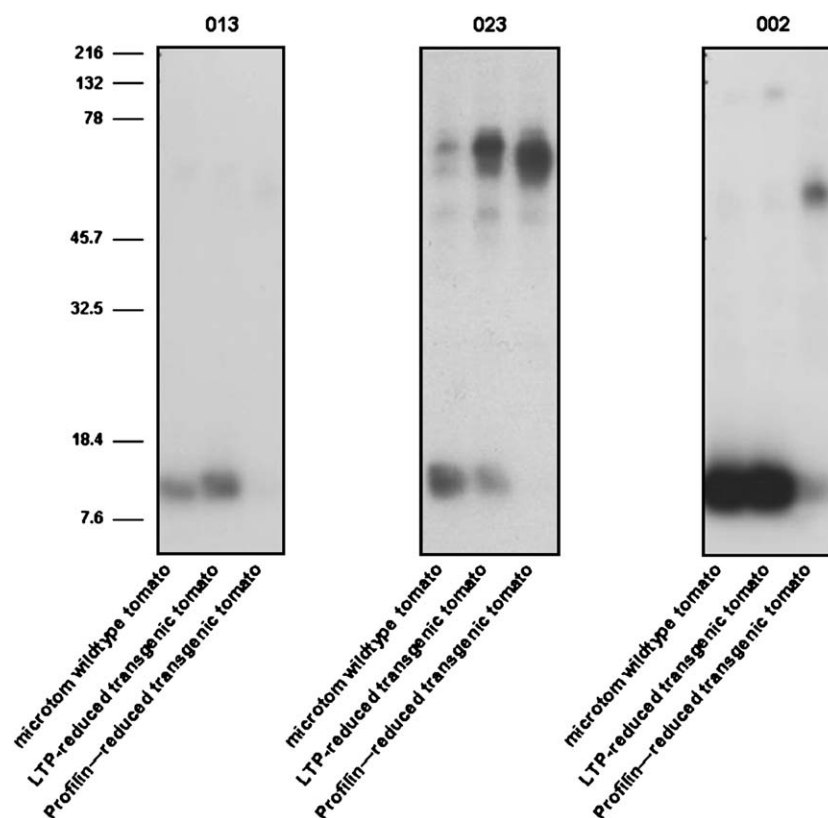


FIG 4. IgE immunoblot of representative patients with tomato allergy (013, 023, and 002). IgE binding to SDS-PAGE-separated and nitrocellulose-blotted fruit protein extracts from WT (lane 1), LTP-reduced (lane 2), and profilin-reduced (lane 3) transgenic tomato plants is shown. The molecular weight standard (in kilodalton) is shown at left.

extracts from WT and profilin-reduced fruits, respectively, by means of immunoblotting. It could be shown that the band at 60 kD is present at variable amounts in both WT and profilin-reduced fruits (data not shown). In the tomato fruit extract from the profilin-reduced plant, a remaining faint specific IgE binding to traces of Lyc e 1 in addition to the high-molecular-weight allergens was visible.

Transgenic tomato fruits show reduced allergenic potential in SPTs

In vivo, in the SPTs the wheal-and-flare reactions to Lyc e 1–reduced tomato fruits differed between individual patients with Lyc e 1 allergy and were dependent on the recognition of additional tomato proteins (Fig 5). In patients with exclusive IgE binding to Lyc e 1 (eg, Fig 4, patient 013), a reduction of the wheal reaction of 63% to 100% compared with that seen in WT MicroTom tomato fruit was found (Fig 5). In patients with additional sensitization to further tomato allergens (eg, Fig 4, patients 023 and 002), this effect was less pronounced, and a reduction of only 16% to 25% was found.

DISCUSSION

The prevalence of adverse reactions to tomato in combination with sensitization ranges from 1.5% to up

to 16% among patient populations with food allergy,⁷ indicating that tomato is a relevant allergenic food. Three tomato allergens have been identified thus far and were included in the official allergen list by the International Union of Immunological Societies Allergen Nomenclature Subcommittee.²⁵ Tomato profilin has been described as a minor allergen,³ and the clinical relevance of sensitization to profilin is controversially discussed.^{26,27} IgE sensitization to profilins from food sources was found to be clinically insignificant in a subgroup of patients with birch pollen allergy because strong IgE reactivity to profilins from cherry, celery, and pear were found in Bet v 2–sensitized subjects without clinical food allergy.²⁸ In addition, neither positive SPT nor *in vitro* results indicated whether a sensitization to cherry profilin in patients with birch pollen allergy correlated with a clinically manifested cherry allergy.²⁹ Interestingly, in a study by Bolhaar et al,³⁰ birch pollen profilin triggered almost no histamine release in a patient with an adverse reaction to Sharon fruit and sensitization to profilin. On the other hand, tomato profilin was capable of inducing mediator release from human basophils, indicating the potential to account for clinical symptoms.³ Moreover, profilins are major allergens in orange fruits, and patients with orange allergy were frequently mono-sensitized to orange profilin. In these patients profilins have the capacity to trigger strong mediator release from

	013	023	002
Histamine			
Sodium chloride			
Wild-type			
Profilin-reduced			
	Mean wheal diameter [mm]	Mean wheal diameter [mm]	Mean wheal diameter [mm]
Histamine	8.5	8.5	7
Sodium chloride	0	0	0
Wild-type	9.5	6	6
Profilin-reduced	3.5	4.5	5

FIG 5. Results of SPTs of Lyc e 1-sensitive patients with tomato allergy (013, 023, and 002) using native tomato fruit from untransformed control (WT) and profilin-reduced tomato fruits.

effector cells.³¹ In summary, it is likely that despite the fact that an IgE response to profilins is considered to be of low significance in some patient populations, there is strong evidence that profilins might indeed be responsible for eliciting food allergy in a subgroup of sensitized patients and specific clinical syndromes.

In an attempt to reduce the allergenicity of tomato fruits, Lyc e 1 expression was efficiently decreased by means of constitutive RNAi. Lyc e 1 encodes profilin with proposed positive and negative functions in actin polymerization. Modulation of its activity in *Arabidopsis thaliana* clearly demonstrated the involvement of profilin in cell elongation, cell shape maintenance, and control of flowering time.¹³ Profilin-deficient *A thaliana* plants showed a dwarf and early flowering phenotype. Similar to *A thaliana*, transgenic tomato plants with strongly reduced profilin accumulation are characterized by a dwarf phenotype. However, flowering time was delayed in profilin-deficient tomato lines, which contrasts the observation made in *A thaliana*. This difference might be explained by tomato being a day-neutral and *A thaliana* being a long-day plant. Despite this negative effect on plant growth, transgenic tomato fruits with strongly decreased accumulation of profilin could be generated and used for SPTs.

Whereas in patients with tomato allergy monosensitized to Lyc e 1 a marked reduction (63% to 100%) of the wheal reaction could be observed in the SPT with transgenic profilin-reduced tomato fruits compared with the WT fruits, this effect was less pronounced (16% to 25%) in patients polysensitized to several tomato allergens. In polysensitized individuals specific IgE to several tomato allergens can contribute collectively to the induction of clinical symptoms, requiring a component-resolved approach^{29,32} for diagnosis and consecutive RNAi silencing. As shown in Fig 4, polysensitized patients (023 and 002) responded to a variety of high-molecular-weight protein

bands in tomato fruit extracts, which might explain the only moderate reduction in wheal diameter.

Furthermore, because of the essential cellular function of profilin, constitutive silencing failed to result in complete profilin knockout plants. Therefore the failure to obtain complete suppression of immune reactivity to Lyc e 1-silenced tomato fruits can additionally be explained by some residual profilin protein accumulating in transgenic tomato fruits (approximately 10% of WT level). In an attempt to inhibit profilin expression more efficiently, an approach to silence its expression in a fruit-specific manner was undertaken (data not shown). To this end, a plant transformation vector carrying a chimeric profilin RNAi construct under the control of a fruit-specific (patatin B33) promoter was constructed and used for tomato transformation. Screening tomato fruits for profilin accumulation yielded fruits with decreased profilin accumulation. However, inhibition of profilin accumulation was inefficient, with more than 20% of WT levels accumulating in fruits of transgenic plants (data not shown). This lack of success in obtaining efficient silencing of profilin by using a fruit-specific promoter can be explained on the basis of temporal and spatial differences in the expression of the transgene and of the endogenous gene. Although Lyc e 1 RNA is detectable throughout fruit development and within the peel, transgene expression decreased during development and was absent in the peel.

In conclusion, we have demonstrated that constitutive RNAi can be used to decrease the allergenic potential of tomato fruits. This approach constitutes a future trend in allergen avoidance. However, to create hypoallergenic tomatoes, the described strategy needs to be extended. Because most patients with tomato allergy are sensitized to several tomato allergens, a number of allergenic proteins must be targeted at the same time. Some of the known allergenic proteins (ie, invertase and profilin) fulfill essential cellular functions, and therefore silencing approaches will have to be combined with the expression of hypoallergenic variants of the same protein to restore physiologic function. In case of profilin, this might be achieved by expression of yeast profilin, which is supposed to be nonallergenic for human subjects.

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