

Yeast profilin complements profilin deficiency in transgenic tomato fruits and allows development of hypoallergenic tomato fruits

Lien Q. Le,^{*} Vera Mahler,[†] Stephan Scheurer,[‡] Kay Foetisch,[‡] Yvonne Braun,[‡] Daniela Weigand,[‡] Ernesto Enrique,[§] Jonas Lidholm,^{||} Kathrin E. Paulus,^{*,1} Sophia Sonnewald,^{*} Stefan Vieths,[‡] and Uwe Sonnewald^{*}

^{*}Department of Biology, and [†]Department of Dermatology, University Hospital Erlangen, Friedrich-Alexander University Erlangen-Nuremberg, Erlangen, Germany; [‡]Division of Allergology, Paul-Ehrlich-Institut, Langen, Germany; [§]Hospital General de Castellón, Castellón, Spain; and ^{||}Phadia AB, Research and Development, Uppsala, Sweden

ABSTRACT Gene silencing of *Lyc e 1* leads to reduced allergenicity of tomato fruits but impaired growth of transgenic tomato plants. The aim of the study was to restore growth of *Lyc e 1*-deficient tomato plants while retaining reduced allergenicity by simultaneous complementation of profilin deficiency by expression of nonallergenic yeast profilin. Transgenic plants were generated and tested by RT-PCR and immunoblotting; allergenicity of yeast profilin and transgenic fruits was investigated by IgE binding, basophil activation, and skin-prick tests. *Lyc e 1* content of transgenic tomato fruits was <5% of that of wild-type plants, causing significantly reduced IgE antibody binding. Simultaneous coexpression of yeast profilin restored growth and biomass production almost to wild-type levels. Yeast profilin, sharing 32.6% amino acid sequence identity with *Lyc e 1*, displayed low IgE-binding capacity and allergenic potency. Among 16 tomato-allergic patients preselected for sensitization to *Lyc e 1*, none showed significant reactivity to yeast profilin. Yeast profilin did not induce mediator release, and coexpression of yeast profilin did not enhance the allergenicity of *Lyc e 1*-reduced fruits. Simultaneous coexpression of yeast profilin allows silencing of tomato profilin and generation of viable plants with *Lyc e 1*-deficient tomato fruits. Therefore, a novel approach to allergen avoidance, genetically modified foods with reduced allergen accumulation, can be generated even if the allergen fulfills an essential cellular function in the plant. In summary, our findings of efficiently complementing profilin-deficient tomato plants by coexpression of low allergenic yeast profilin demonstrate the feasibility of creating low-allergenic food even if the allergen fulfills essential cellular functions.—Le, L. Q., Mahler, V., Scheurer, S., Foetisch, K., Braun, Y., Weigand, D., Enrique, E., Lidholm, J., Paulus, K. E., Sonnewald, S., Vieths, S., Sonnewald, U. Yeast profilin complements profilin deficiency in transgenic tomato fruits and allows development of hypoallergenic tomato fruits. *FASEB J.* 24, 000–000 (2010). www.fasebj.org

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FOOD ALLERGY HAS A REMARKABLE prevalence in the general population and may affect 3–4% of adults and up to 6% of children (1). Since specific immunotherapy is not established for food allergy, symptoms are treated mainly by systemic medication or avoided by the elimination of the offending food (1, 2). However, strict elimination diets may lead to malnutrition and social deprivation. Thus, development of foods with reduced allergen content would significantly improve the quality of life of patients with food allergies.

The conventional method to produce hypoallergenic food involves enzymatic or physical processing of allergenic proteins. Another approach is allergen suppression by RNA interference (RNAi) technology. This technique has successfully been used to reduce the allergenic potential of apples, tomatoes, and peanuts (3–7). In our previous studies (4–6), 2 tomato (*Lycopersicon esculentum*) allergens, *Lyc e 1* [profilin (PFN)] and *Lyc e 3* (nonspecific lipid transfer protein), were successfully silenced by RNAi. Both *in vitro* and *in vivo* tests with tomato-allergic patients revealed a strong reduction in allergenic potential of transgenic tomato fruits. However, while *Lyc e 3*-silenced plants showed normal growth (6), severe growth retardations were observed in *Lyc e 1*-silenced plants (5). These plants were smaller, exhibited delayed flowering, and formed fewer fruits compared with nontransformed control plants. This observation suggested that a bottleneck for creating hypoallergenic foods may be the abrogation of essential cellular functions of certain target allergens (8).

¹ Correspondence: Friedrich-Alexander Universität Erlangen-Nürnberg, Lehrstuhl für Biochemie, Staudtstr. 5, 91058 Erlangen, Germany. E-mail: kpaulus@biologie.uni-erlangen.de
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PFNs are ubiquitous proteins of 12–15 kDa present in all eukaryotic cells (9), where they are involved in polymerization and sequestration of actin. Although the sequence similarity between PFNs from organisms of different kingdoms (animals, fungi, and plants) is low, they share a conserved 3-dimensional structure (10). Based on substantial sequence similarities of PFNs among flowering plants, IgE cross-reactivity between PFNs from pollen, plant food, and natural rubber latex can be observed, and PFNs have been classified as plant pan-allergens (11, 12). Reduction in PFN content, both in tomato and *Arabidopsis* plants, results in negative growth effects, indicating that PFN is essential for normal plant development (5, 13, 14). It is well documented that plant PFNs share functional similarities to nonplant PFNs and can complement PFN deficiencies in mammalian, yeast, and *Dictyostelium discoideum* cells (15–18). Therefore, it is tempting to speculate that nonplant PFNs might be able to substitute for PFN deficiency in PFN-silenced plants.

The aim of this study was to complement Lyc e 1-deficient tomato plants by a nonplant PFN to examine whether the physiological function of PFN could be restored without introducing a novel allergen. Yeast profilin (PFY1) from baker's yeast (*Saccharomyces cerevisiae*) has not been described as an allergen so far, and therefore was chosen to complement Lyc e 1 deficiency in transgenic tomato plants. Here, we show that PFY1 does not exhibit allergenic potency to tomato-allergic patients sensitized to Lyc e 1. Furthermore, the severely compromised phenotype of Lyc e 1-silenced tomato plants could be restored by overexpression of PFY1. Both *in vitro* and *in vivo* assays revealed that the PFY1 substitution did not increase the allergenic potential of transgenic tomato fruits. Therefore, using PFN as model, we suggest that the substitution of an essential, but allergenic, protein with a functional, nonallergenic variant might be a useful approach to produce hypoallergenic foods.

MATERIALS AND METHODS

Patients and sera

Patients with tomato allergy were selected on the basis of their clinical history (*e.g.*, oral allergy syndrome, gastrointestinal or contact urticaria on ingestion of tomato fruits) and positive response in prick-to-prick tests performed with tomato fruits or *in vitro* basophil histamine release assays. Sensitization to tomato allergens of these patients was confirmed by detection of tomato-specific serum IgE by ImmunoCAP (Phadia, Uppsala, Sweden) or IgE immunoblotting. IgE reactivity to purified Lyc e 1 was monitored by immunoblotting and experimental ImmunoCAPs. Sera from nonallergic individuals served as negative controls. Patients were recruited in Germany and Spain. Approval of the local ethics committees (Faculty of Medicine, University of Erlangen-Nuremberg, No. 3344; and General Hospital, Castellón, Spain) for this study and written informed consent of all participants were obtained.

cDNA cloning and expression of tomato and PFY1

For immunological analysis (IgE-immunoblot, ImmunoCAP, ELISA inhibition assay, and mediator release assay), the recombinant tomato PFN (Lyc e 1.02 isoform, acc. no. AY061819) and PFY1 were expressed in *Escherichia coli* and purified by affinity chromatography. The coding regions of Lyc e 1.02 and PFY1 were cloned into the pET-45b (+) vector containing an N-terminal His-tag (Novagen, Darmstadt, Germany) using the following specific oligonucleotides: 5' Lyc e 1.02: 5'-AGGTACCTCGTGGCAAACATATG-3', 3' Lyc e 1.02: 5'-AGTCGACCTAGAGACCCTGTTCAATG-3', 5' PFY13: 5'-AGGTACCTCTTGGCAA GCATACAC-3', 3' PFY13: 5'-AGGATCCTTAGTATTGAACACCAATC-3'. Expression constructs were transformed in *E. coli* strain Rosetta (DE3). Protein synthesis was induced with 0.5 mM IPTG at $OD_{600} = 0.5$ for 4 h at 37°C. Purification of the expressed His-tag protein was done by Ni-NTA agarose chromatography (Qiagen, Hilden, Germany) following the manufacturer's instructions. Structural integrity was proven by circular dichroism (CD) spectroscopy. CD spectra of purified recombinant PFNs (20.0 μ M) were recorded in 10 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.4) on a J-810 S spectropolarimeter (Jasco, Gross-Umstadt, Germany) with constant N_2 flushing at 20°C. The measurement was performed in a quartz glass cuvette (1 mm) with a step width of 1 nm and a band width of 1 nm. The spectral range was 180–260 nm at 50 nm/min. Ten scans were accumulated, and buffer spectra were subtracted. The results are expressed as mean residue molar ellipticity $[\Theta]MRD$, with θ , observed ellipticity (mdeg); N , number of amino residues; d , optical path length (cm); and c , protein concentration (mM): $[\Theta]MRD = 100 \times \theta / N \times c \times d$ (see Supplemental Data).

Analysis of total protein by SDS-PAGE, IgE, and IgG immunoblotting

For IgG immunoblotting, total protein was extracted from wild-type (WT) and transgenic tomato fruits (~1 g fresh weight), separated on SDS-PAGE (T: 15%, v/v) and blotted onto nitrocellulose membranes (Macherey-Nagel, Düren, Germany). Membranes were blocked for 2 h in 5% skimmed milk/TBST (20 mM Tris, pH 7.0; 500 mM NaCl; and 0.1% Tween 20), and incubated overnight at 4°C with rabbit antiserum raised against the myc protein (diluted 1:3000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or anti-ragweed profilin antiserum (diluted 1:10,000). ECL immunodetection using anti-rabbit antibodies (Amersham Pharmacia Biotech, Braunschweig, Germany) was performed according to the manufacturer's instructions. Purified rLyc e 1 and rPFY1 (1 μ g/cm) were tested for IgE reactivity using sera (diluted 1:10 in PBS) from tomato-allergic patients. (see Supplemental Data).

Experimental ImmunoCAPs

Experimental ImmunoCAP tests carrying allergens Lyc e 1 or PFY1 were prepared according to Marknell DeWitt *et al.* (20), and assays were performed using an ImmunoCAP 250 instrument (Phadia) according to the manufacturer's instructions. Statistical data are represented as a box plot (SigmaPlot 11.0; Systat Software, Inc., San Jose, CA, USA). The boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Error bars above and below the box indicate the 90th and 10th percentiles.

Tandem vector construction

To prepare a construct for transgenic expression of PFY1 in tomato plants, the coding region of PFY1 (acc. no. M23369)

was amplified by PCR using the following gene-specific primers: 5'PFY1: 5'-GGTACCATTATGTCTTGGCAAGCATACACTGATAACTTAATG-3', 3'PFY2: 5'-AGGATCCGTATTGAACACCAATCAAGTAGTC-3'. The PCR product lacking the stop codon was fused to the triple-myc tag and cloned into the binary plant transformation vector pBinAR (19) between the constitutive 35S promoter and the octopine synthase (OCS) terminator, yielding plasmid pBin_35S_PFY1myc. Subsequently, the entire chimeric gene (35S promoter, PFY1, triple myc, and OCS) was PCR amplified using the following oligonucleotides: 5'PFY3, 5'-AAAGCTTGAATTCCCTATGAGTCAAAG-3'; and 3'PFY4, 5'-AAAGCTTGGACAATCAGTAAATTGAACG-3'.

The PCR product was cloned into the pCR blunt vector (Invitrogen, Carlsbad, CA, USA) and subjected to sequence analysis. Subsequently, a fragment containing the entire open reading frame of PFY1 together with 35S promoter, triple myc, and OCS terminator was excised using *HindIII* restriction sites and ligated into the pGWPFN_RNAi (5) digested by *HindIII*. The pGWPFN_RNAi plasmid was used to silence Lyc e 1 in transgenic tomato plants (5). The resulting tandem vector construct was designated as pGWPFN_RNAi/PFY1 (see Fig. 5A) and used for *Agrobacterium*-mediated transformation of tomato plants.

Plant transformation and growing condition

The pGWPFN_RNAi/PFY1 construct was transformed into *Agrobacterium tumefaciens* CV58C1, carrying the virulence plasmid pGV2260 and used for *Agrobacterium*-mediated transformation of tomato plants (cv. Microtom). Tomato cotyledon explants were transformed and regenerated as described by Ling *et al.* (21). Transgenic Lyc e 1-silenced (Lyc1_RNAi), PFY1 coexpressing (Lyc1_RNAi/PFY1), and WT tomato plants (cv. Microtom) were cultivated as described by Le *et al.* (5). Green biomass was assessed in 10-wk-old tomato plants.

RNA isolation and RT-PCR based quantification of PFN mRNA expression in transgenic plants

The mRNA transcript expression level of Lyc e 1 in the Lyc e 1-silenced lines 14.2, 15.6, and 21.7 (T2 generation) and the PFY1-expressing plant lines 28, 53, and 71 (T1 generation)

was evaluated using RT-PCR. To this end, total RNA was isolated from leaves of 10-wk-old tomato plants as described by Logemann *et al.* (22).

ELISA inhibition assay

Ninety-six-well Maxisorb plates (Nunc, Wiesbaden, Germany) were coated overnight at 4°C with 0.5 µg of rLyc e 1.02 diluted in PBS. The plates were blocked with PBST (PBS plus 0.05% Tween 20) supplemented with 1% BSA for 2 h. After 3 washing steps with PBST, each well was incubated with 100 µl diluted serum (1:10 with PBS) from a tomato-allergic patient sensitized to Lyc e 1, combined with either 100 µl buffer or with 100 µl tomato extracts (0.01, 0.1, 1.0, 10, and 100 µg/ml of total protein) for 1 h. As positive control rLyc e 1 (10 µg/ml) was applied as inhibitor. Subsequently, plates were washed and incubated with a horseradish peroxidase-coupled monoclonal anti-human-IgE antibody (Southern Biotechnology Associates Inc., Birmingham, AL, USA) for 1.5 h. After 3 additional washing cycles, visualization was performed with tetramethylbenzidine and hydrogen peroxide for 5–10 min. The reaction was stopped with 50 µl of 3 M H₂SO₄, and absorption was measured at 450 nm. Duplicate determinations were performed for each sample. Inhibition rates were calculated as a percentage of noninhibitor values.

Basophil histamine release assay

The basophil histamine release assay was performed with stripped and passively sensitized basophils from a nonallergic donor as described previously (23). For stimulation of the cells, purified allergens and tomato extracts were applied in final concentrations ranging from 0.1 ng/ml to 10 µg/ml. To reduce the endogenous content of histamine, tomato extracts were dialyzed overnight (4°C) toward PBS buffer in a slide-A-lyzer dialysis cassette (Pierce, Cheshire, UK). Released histamine was measured by a commercial enzyme immunoassay (Immunotech, Marseille, France).

Skin-prick test

Prick-to-prick tests in tomato-allergic patients were performed as described previously (24). Skin tests using tomato fruits

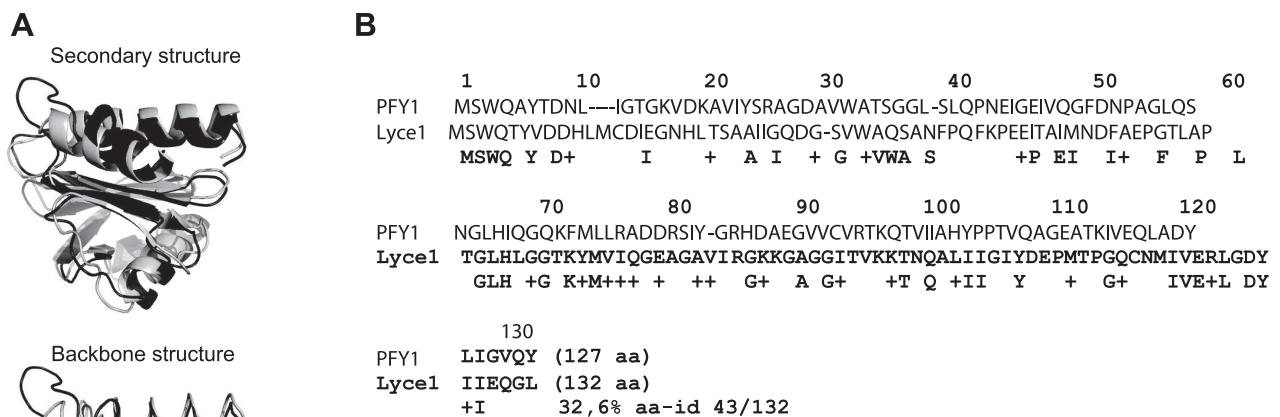
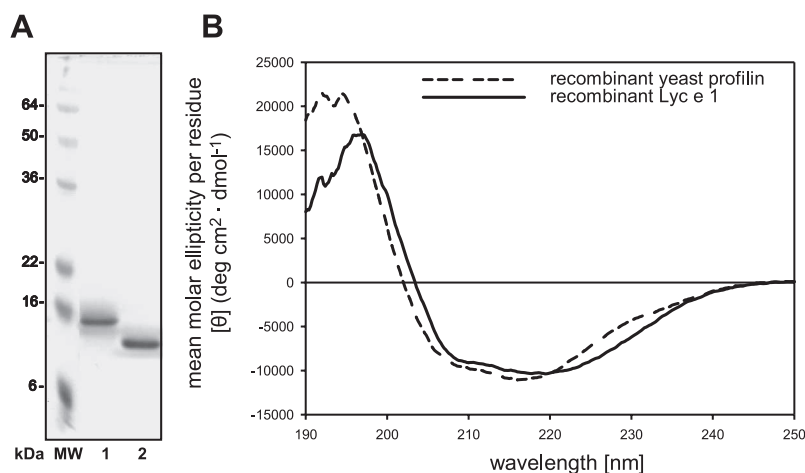


Figure 1. Comparison of PFY1 and Lyc e 1 amino acid sequences and folding structure. A) Ribbon diagram and backbone structure of Lyc e 1.02 (black; acc. no. AY061819) and PFY1 (gray; pdb 1K0K; acc. no. M23369). The model of Lyc e 1 was proposed via the SWISS-MODEL program (<http://swissmodel.expasy.org>). The figure was generated by the PyMOL 0.99β34 program (2006; DeLano Scientific LLC; <http://www.pymol.org>). B) Alignment of the deduced amino acid sequences of Lyc e 1.02 (acc. no. AY061819) and PFY1 (acc. no. M23369). Identical and similar (+) amino acids are indicated.

Figure 2. Purification and CD spectroscopy of recombinant PFY1 and Lyc e 1. *A*) SDS-PAGE and Coomassie staining of purified rLyc e 1 (lane 1) and rPFY1 (lane 2). *B*) CD spectra of purified recombinant yeast and tomato PFNs recorded on a J-810 S spectropolarimeter (Jasco).



from cv. Microtom WT, PFN reduced, and PFY1 complemented fruits (after depletion of seeds) were repeated three times. Control prick-test solutions (histamine diluted 1:1000 and 0.9% sodium chloride) were purchased from Allergopharma (Reinbek, Germany). Mean wheal diameters were determined after 20 min, and sizes ≥ 3 mm were considered positive.

RESULTS

PFY1 displays almost no IgE reactivity in tomato-allergic patients sensitized to tomato PFN

Despite a low amino acid sequence identity of 32.6% between the 2 PFNs, the proposed 3-dimensional model of Lyc e 1 (acc. no. AY061819) is similar to that of PFY1 (pdb 1K0K, acc. no. M23369; **Fig. 1**). The Lyc e 1 model was generated by the Swiss-Model program (Swiss Institute of Bioinformatics, Biozentrum, University Basel; <http://swissmodel.expasy.org>) using structures of PFNs from rubber tree (pdb 1G5U, Hev b 8), birch (pdb 1CQA, Bet v 2), and *Arabidopsis* (pdb 3NUL) as a template. Both PFNs reveal 7 β -sheets, 2 parallel α -helices on 1 side, and 1 α -helix on the other side of the molecule. Remarkably, the cDNA alignment showed 21% identity, with stretches of only up to 9

identical nucleotides (see Supplemental Fig. 1). Both his-tagged PFNs were prepared with a high degree of purity showing an apparent molecular mass of 14 kDa for Lyc e 1 and 12 kDa for PFY1 (**Fig. 2A**). Comparative CD spectroscopy indicated similar folding of the 2 proteins (**Fig. 2B**). To exclude that the transgene is allergenic, rLyc e 1 and rPFY1 were compared for IgE-binding capacity. Immunoblotting using sera from 16 tomato-allergic patients with confirmed sensitization to rLyc e 1 (**Fig. 3**) showed IgE binding to rLyc e 1, whereas no or at most marginal IgE reactivity to rPFY1 was detectable in some sera. Both PFNs were detected by a cross-reactive rabbit antiserum, demonstrating their immune reactivity. To further substantiate the safety of PFY1 expression in transgenic plants, all patients sera were quantified for specific IgE to PFY1 using the ImmunoCAP assay system (**Fig. 4**). The IgE binding to rLyc e 1 was confirmed for 15 of the 16 patients. Low levels of IgE to Lyc e 1 (0.59, 0.49, and 0.29 kU_A/L) in 3 sera correlated with weak IgE binding in immunoblot analysis (**Fig. 3**, patients 5, 8, and 9). In contrast, the majority of sera displayed no IgE binding to PFY1. Detectable IgE to PFY1 was observed in sera from 5 patients but at very low levels (0.38–0.47 kU_A/L) as compared with IgE binding to Lyc e 1 (1.79–7.47 kU_A/L). Taking into account all sera tested,

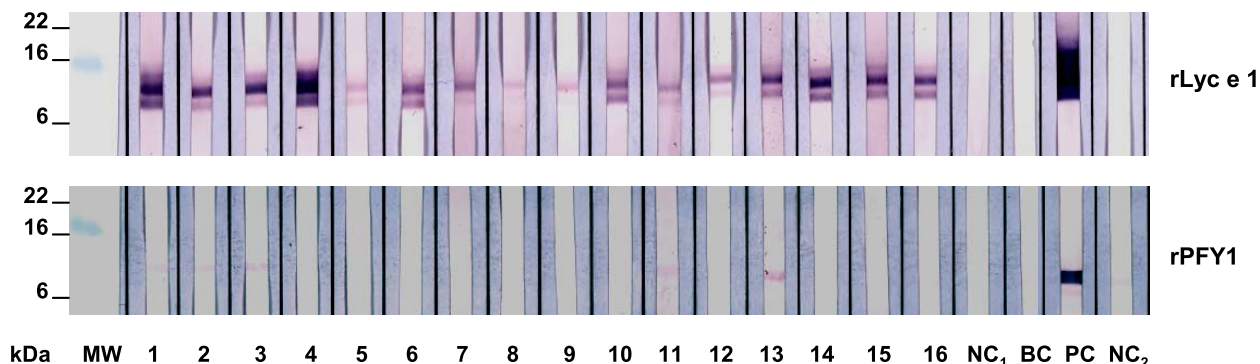


Figure 3. IgE-binding analysis of recombinant PFY1 and Lyc e 1. IgE-reactivity of rLyc e 1 (top panel) and rPFY1 (bottom panel). Lanes 1–16, selected sera from tomato-allergic patients. NC₁, nonallergic control; BC, buffer control; PC, rabbit anti-ragweed PFN; NC₂, rabbit normal serum.

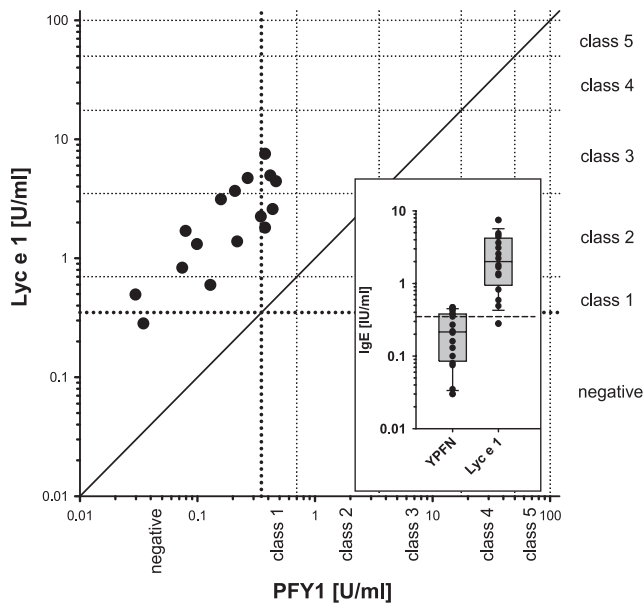


Figure 4. Quantification of Lyc e 1 and PFY1 specific IgE. Determination of IgE binding to purified rLyc e 1 and rPFY1 by experimental ImmunoCAPs using 16 tomato-allergic patients selected by IgE-reactivity to Lyc e 1. Cutoff value of 0.35 kU_A/L for positive ImmunoCAP testing is indicated. Statistical values are represented as a box plot (SigmaPlot 11.0) indicating the median IgE values.

the median specific IgE concentration was 2.00 kU_A/L for Lyc e 1 and 0.215 kU_A/L for PFY1.

Generation of Lyc e 1-deficient, PFY1-expressing transgenic tomato plants

To study whether expression of PFY1 is sufficient to complement Lyc e 1 deficiency, a total of 80 kanamycin-resistant tomato plants (referred to as Lyc e 1_RNAi/

PFY1 plants) was analyzed by immunoblotting using an anti-myc antibody for the detection of myc-tagged PFY1 and a cross-reactive anti-PFN antibody recognizing Lyc e 1 (**Fig. 5**). Three lines (Lyc e 1_RNAi/PFY1 transgenic lines 28, 53, and 71) were selected for further analysis due to the observed reduction in Lyc e 1 content and concurrent expression of PFY1 and compared with 3 transgenic lines with strong reduction of Lyc e 1 (Lyc e 1_RNAi lines 14, 15, and 21). With the use of an anti-ragweed PFN antibody, a strong protein band migrating at 14 kDa in protein extracts from WT fruits was detected (**Fig. 5B**). A weak Lyc e 1 protein band could be visualized in line 14, while no Lyc e 1 accumulation was detectable in lines 15 and 21. Among the selected PFY1-expressing plants, line 28 exhibited a strong reduction in Lyc e 1 compared with WT while no residual Lyc e 1 could be detected in lines 53 and 71 (**Fig. 5B**). Immunoreactivity to the myc-tag indicated the simultaneous expression of the PFY1 fusion protein with an apparent molecular mass of 18 kDa in Lyc e 1_RNAi/PFY1 transgenic lines, predominantly in lines 53 and 71, but not in WT and Lyc e 1_RNAi lines.

PFY1 complements the growth phenotype of Lyc e 1-deficient tomato plants

To analyze whether expression of PFY1 in tomato plants can overcome the severe growth defects observed in Lyc e 1-deficient tomato plants, accumulation of green biomass was determined and related to the amount of tomato PFN mRNA. As shown in **Fig. 6B**, the amount of Lyc e 1 transcripts varied in WT plants between 55–100%, while it ranged from <1% to nearly 100% in the segregating transgenic populations. Since growth of Lyc e 1-silenced plants containing >5% residual Lyc e 1 transcript showed no significant difference to WT plants, biomass production of PFY1-express-

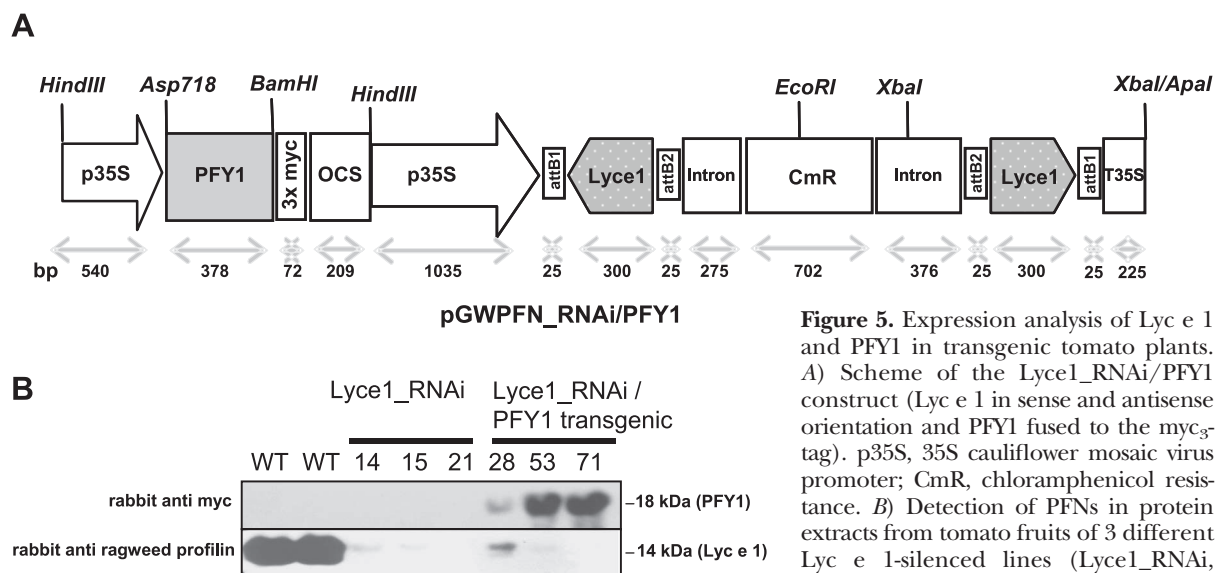


Figure 5. Expression analysis of Lyc e 1 and PFY1 in transgenic tomato plants. A) Scheme of the Lyc e 1_RNAi/PFY1 construct (Lyc e 1 in sense and antisense orientation and PFY1 fused to the myc₃-tag). p35S, 35S cauliflower mosaic virus promoter; CmR, chloramphenicol resistance. B) Detection of PFNs in protein extracts from tomato fruits of 3 different Lyc e 1-silenced lines (Lyc e 1_RNAi, lines 14, 15, and 21; ref. 5) and 3

different PFY1-expressing lines (Lyc e 1_RNAi/PFY1, lines 28, 53, and 71) by immunoblot analysis using a rabbit serum raised against the myc-tag for detection of PFY1 and a rabbit serum raised against ragweed PFN for detection of Lyc e 1. PFY1 and Lyc e 1 migrated at ~18 and 14 kDa, respectively.

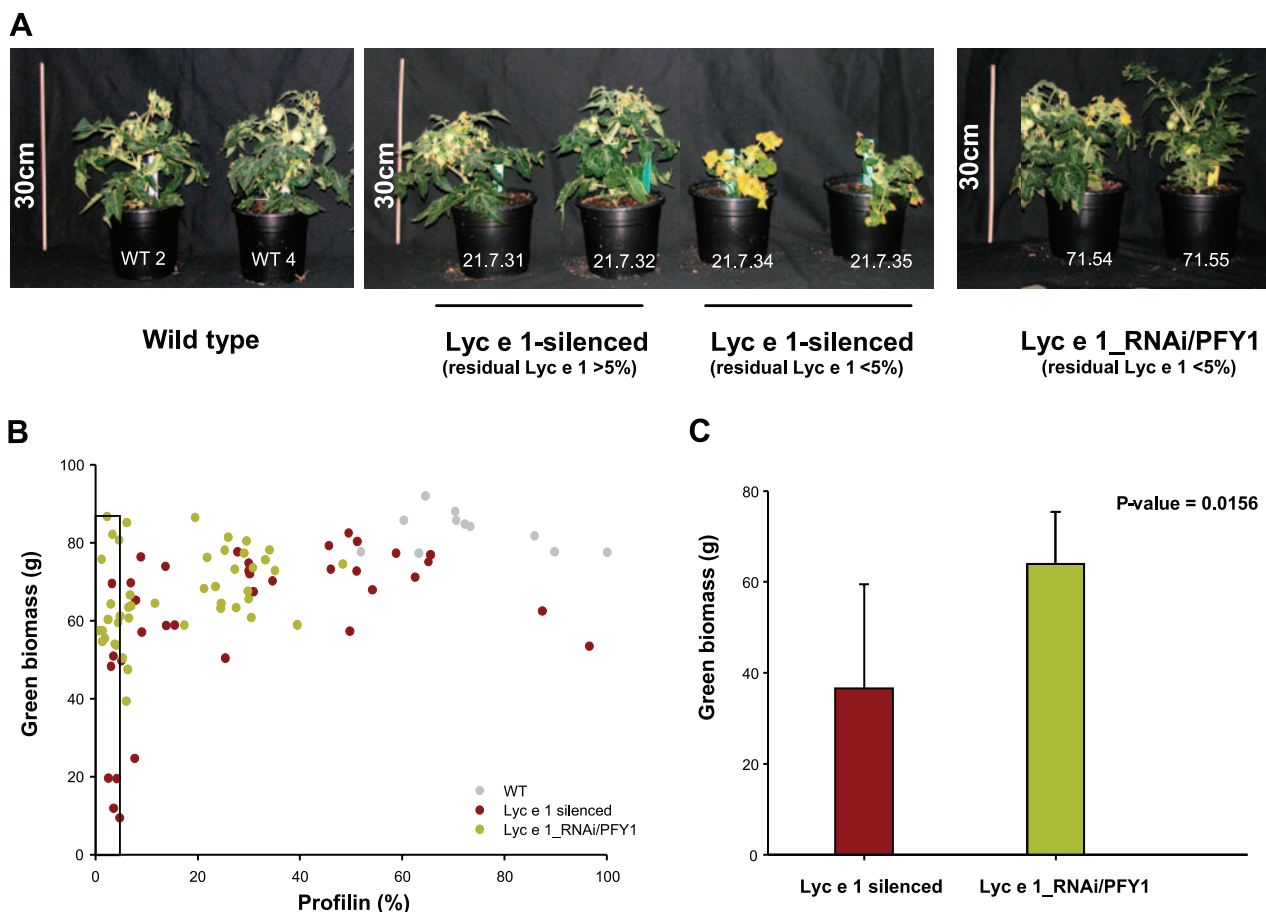


Figure 6. Correlation between green biomass and residual Lyc e 1 transcript abundance. **A)** Phenotype of 10-wk-old WT, Lyc e 1-silenced, and Lyc e 1-silenced and simultaneously PFY1-expressing lines exhibited WT-like phenotypes. Dwarf phenotypes were observed in Lyc e 1-silenced plants containing <5% remaining Lyc e 1. **B)** Correlation between green biomass and residual Lyc e 1 transcript accumulation. Lyc e 1 was amplified by RT-PCR and quantified using the ImageJ software. Tomato plants containing <5% residual Lyc e 1 are enclosed in the box. **C)** Comparison of the green biomass produced by Lyc e 1-silenced and Lyc e 1_RNAi/PFY1 tomato plants with <5% remaining Lyc e 1 transcripts as compared with WT controls ($P=0.0156$).

ing and Lyc e 1 reduced plants containing <5% residual Lyc e 1 transcript was compared (Fig. 6C). The mean green biomass for Lyc e 1-silenced and PFY1-complemented plants was 36.61 ± 22.87 and 63.98 ± 11.5 g ($P=0.0156$), respectively. Thus, PFY1-complemented plants showed a 1.7-fold higher biomass accumulation than Lyc e 1-silenced plants. In comparison to nontransformed controls, Lyc e 1_RNAi plants attained 44.2% biomass, while Lyc e 1_RNAi/PFY1 plants reached 77.1%.

ELISA inhibition assays confirm silencing of Lyc e 1 in transgenic tomato fruits and lack of cross-reactivity between PFY1 and Lyc e 1

Dose-dependent IgE inhibition assays were performed to verify the suppression of tomato PFN as well as the absence of IgE cross-reactivity of plant-produced PFY1. A representative serum of a patient (Fig. 3, patient 4) who had a history of tomato allergy, exhibited a positive ImmunoCAP response to tomato ($15.5 \text{ kU}_A/\text{L}$), and showed IgE sensitization to Lyc e 1 ($7.47 \text{ kU}_A/\text{L}$) and PFY1 ($0.38 \text{ kU}_A/\text{L}$), was applied. Recombinant PFY1, rLyc

e 1, and extracts from fruits of WT and 3 Lyc e 1_RNAi/PFY1 transgenic lines were used as inhibitors (Fig. 7A). After preincubation of serum with extracts from tomato WT fruits ($100 \text{ } \mu\text{g}/\text{ml}$) or rLyc e 1 ($10 \text{ } \mu\text{g}/\text{mL}$), IgE binding to Lyc e 1 was completely inhibited (Fig. 7A). In contrast, the IgE binding to rLyc e 1 was not affected by serum preincubation with rPFY1 or fruit extracts from PFY1-expressing plants, indicating that fruit-produced PFY1 is not IgE reactive. Moreover, transgenic fruits displayed a strong reduction of Lyc e 1 accumulation, suggesting that the remaining Lyc e 1 in Lyc e 1_RNAi/PFY1 transgenic fruits is very low. This confirms that when the tandem expression vector pGWPFN_RNAi/PFY1 was expressed, the PFY1 accumulates while Lyc e 1 levels decline in transgenic tomato plants.

Transgenic fruits exhibit a reduced allergenic potential as shown by *in vitro* histamine release assays and *in vivo* skin-prick tests

For *in vitro* histamine release assays, removal of endogenous histamine in tomato extracts was confirmed by

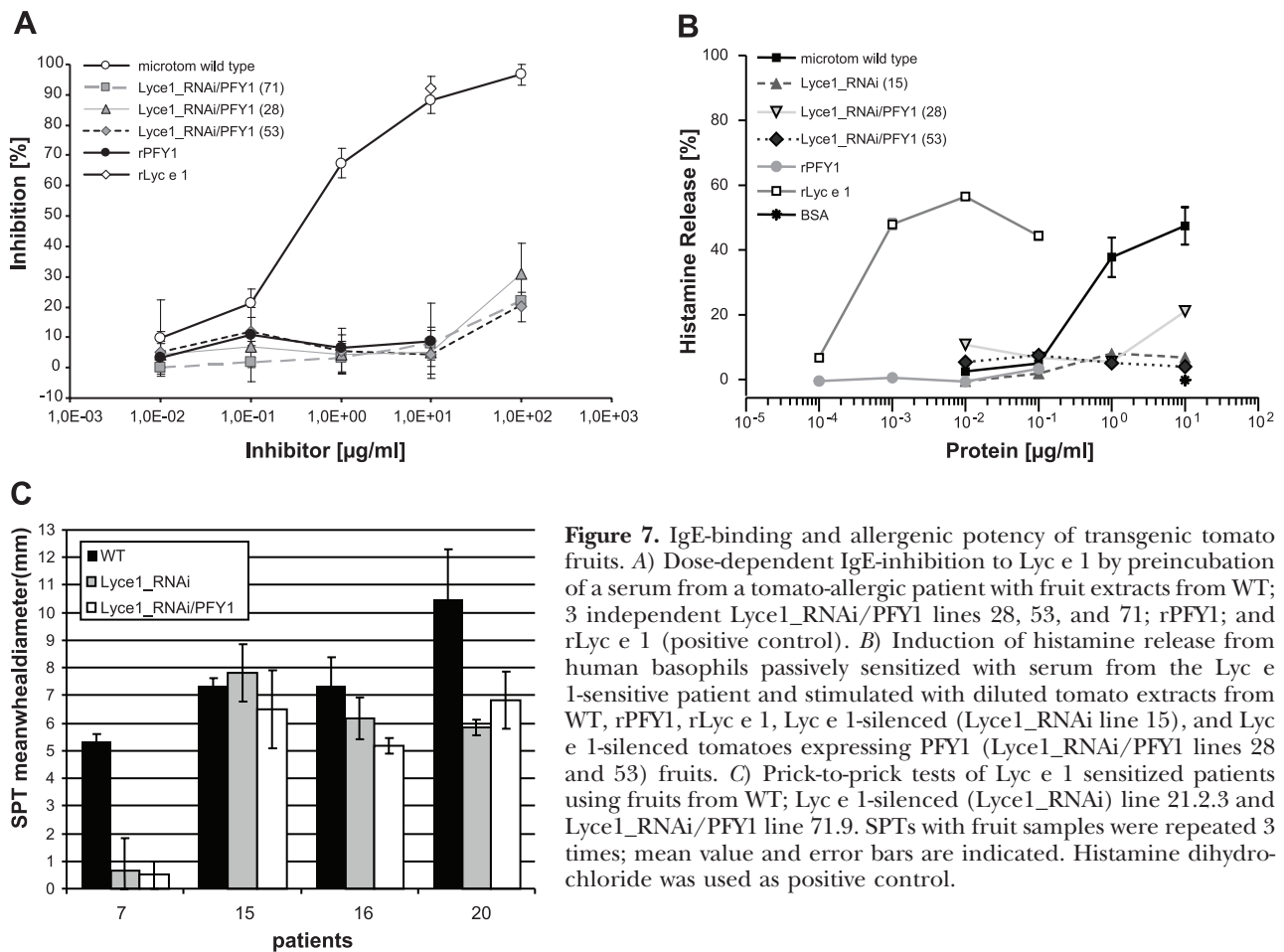


Figure 7. IgE-binding and allergenic potency of transgenic tomato fruits. **A)** Dose-dependent IgE-inhibition to Lyc e 1 by preincubation of a serum from a tomato-allergic patient with fruit extracts from WT; 3 independent Lyce1_RNAi/PFY1 lines 28, 53, and 71; rPFY1; and rLyc e 1 (positive control). **B)** Induction of histamine release from human basophils passively sensitized with serum from the Lyc e 1-sensitive patient and stimulated with diluted tomato extracts from WT, rPFY1, rLyc e 1, Lyc e 1-silenced (Lyce1_RNAi line 15), and Lyc e 1-silenced tomatoes expressing PFY1 (Lyce1_RNAi/PFY1 lines 28 and 53) fruits. **C)** Prick-to-prick tests of Lyc e 1 sensitized patients using fruits from WT; Lyc e 1-silenced (Lyce1_RNAi) line 21.2.3 and Lyce1_RNAi/PFY1 line 71.9. SPTs with fruit samples were repeated 3 times; mean value and error bars are indicated. Histamine dihydrochloride was used as positive control.

the competitive enzyme immunoassay (not shown). As shown by a representative serum (Fig. 3, patient 4), recombinant Lyc e 1 and fruit extract from WT plants induced a strong release of histamine, reaching a maximum of 60% using 10 ng/ml of rLyc e 1 and 50% on stimulation with extract from WT (10 μg/ml) (Fig. 7B). The calculated concentration required for half maximal histamine release was 0.5 ng/ml (HR_{50} =30% release) for rLyc e 1 and 0.5 μg/ml (HR_{50} =25% release) for WT fruit extracts. In contrast, rPFY1 and transgenic lines induced almost no histamine release (<10% maximal release), except for the Lyce1_RNAi/PFY1 line 28. The maximal mediator release in response to this line was 20% at the highest concentration applied. Since the remaining Lyc e 1 content in the Lyce1_RNAi/PFY1 line 28 was higher compared with the other 2 transgenic lines, slightly higher mediator release might be due to a residual allergenic potency of this line. In contrast to Lyc e 1, PFY1 was not capable of triggering mediator release using 2 additional sera from tomato-allergic patients sensitized to Lyc e 1 (not shown).

Skin-prick tests were performed in 4 tomato-allergic patients with tomato-specific IgE (f25: 1.33, 4.85, 3.08, and 1.51 kU_A/L) and sensitization to Lyc e 1 (0.83, 4.91, 3.1, and 0.41 kU_A/L) as determined by ImmunoCAP testing (Fig. 7C). Histamine dihydrochloride used as positive control induced in all patients a mean wheal

diameter of 4.5–7 mm (not shown). Prick-to-prick-tests were performed with WT fruits and fruits from the T2 generation of Lyc e 1-silenced plants (Lyce1_RNAi line 21.2.3) and the T1 generation of PFY1 plants (Lyce1_RNAi/PFY1 line 71.9). WT fruits led to wheal diameters ranging from 5.5 to 12 mm. Skin reactivity to Lyc e 1-silenced and PFY1 complemented transgenic fruits was strongly reduced in 2 patients: by 87% (mean wheal diameter of 0.67 mm) and 91% (mean wheal diameter of 0.5 mm) for patient 7, and 44% (mean wheal diameter of 5.83 mm) and 35% (mean wheal diameter of 6.83 mm) for patient 20. Patient 16 showed 16% reduction of skin reactivity to Lyc e 1-silenced and 29% to PFY1-expressing transgenic fruits. For patient 15, skin reactivity was similar to WT. Notably, transgenic expression of PFY1 did not enhance the skin test reaction in any of the patients. All patients were sensitized to additional high-molecular-weight tomato proteins, with a predominant reactivity to a protein with an apparent molecular mass of 45 kDa (not shown). Moreover, IgE binding to bromelain was observed in 2 sera.

In summary, our data suggest that in a subgroup of tomato-allergic patients, both kinds of Lyc e 1-silenced transgenic fruits evaluated in this study (Lyc e 1 silenced with or without PFY1 complementation) demonstrated a reduced *in vivo* allergenic potency as compared with WT fruits.

DISCUSSION

In an attempt to provide proof of concept that food with reduced allergenicity can be produced by genetic engineering, the PFN Lyc e 1 was silenced in transgenic tomato fruits. Constitutive silencing of Lyc e 1 caused severe growth retardation in a number of plants, emphasizing the essential function of PFN (5). This outcome suggested that efficient silencing of Lyc e 1 in transgenic tomato plants would require simultaneous expression of a nonallergenic PFN to maintain biological viability. To minimize the risk of IgE cross-reactivity, a nonplant PFN from yeast (PFY1) was selected to substitute Lyc e 1. PFY1 is encoded by a single copy gene (25). Although PFY1 shares only 32.6% identical amino acids with Lyc e 1, the 2 PFNs display a highly conserved tertiary structure (Fig. 1). Along this line, it has been demonstrated that plant PFNs can complement PFN deficiency in yeast or *D. discoideum* (17, 18). Considering these data, it was expected that expression of PFY1 would be able to complement PFN deficiency in transgenic tomato plants.

Before generating in transgenic tomato plants, recombinant PFY1 and Lyc e 1 were prepared in order to examine their cross-reactive potential. According to SDS-PAGE analysis and CD spectroscopy, recombinant PFY1 and Lyc e 1 were highly pure and contained similar secondary structure elements (Fig. 2). Although the 2 PFNs differ only by 5 amino acids in length, the apparent molecular mass, as determined by SDS-PAGE, was slightly different, a phenomenon that has been described for other members of the PFN family (26). A panel of 16 tomato-allergic patients selected by immunoblotting was included in the study. Whereas almost all patients' sera showed a clear reactivity to rLyc e 1, no or at most marginal IgE reactivity to rPFY1 was detectable in same sera by immunoblotting (Fig. 3). IgE antibody recognition of PFY1 specific IgE was further evaluated for all sera by experimental ImmunoCAP testing (Fig. 4). Sensitization to Lyc e 1 was confirmed for all patients, except for 1 subject showing a weak IgE binding (Fig. 3, patient 9). No IgE binding to PFY1 was detected for almost all sera, except for the sera of 5 patients showing weak class 1 values (<0.47 kU_A/L). Taking into account all sera tested, the median PFY1 specific IgE concentration was >10 -fold lower in comparison to Lyc e 1. These data suggested that expression of PFY1 in transgenic tomato plants would confer a low risk of cross-reactivity and encouraged us to pursue the approach and generate a chimeric vector (Lyc1_RNAi/PFY1) for Lyc e 1 suppression and simultaneous overexpression of PFY1 (Fig. 5).

Comparing transgenic plants with or without simultaneous expression of PFY1 revealed that PFY1 expression led to substantially increased biomass production in Lyc e 1 reduced plants, from ~ 44 to 77% of WT biomass. This partial complementation might be explained by low level of expression of PFY1 in transgenic tomato plants. As estimated from an IgG immunoblot-

ting experiment, the highest concentration of PFY1 (Fig. 5B, lanes 53 and 71) reached only $\sim 40\%$ of the Lyc e 1 concentration in WT plants (not shown).

Having demonstrated the low IgE-binding activity of PFY1 and its ability to reconstitute the phenotype of transgenic tomato plants, we investigated the allergenic potency by *in vitro* and *in vivo* mediator release tests. The ability to induce histamine release of rPFY1 was >1000 -fold lower as compared with rLyc e 1 (Fig. 7B). Although baker's yeast has been implicated as a cause of certain allergic reactions (27, 28) and IgE binding to yeast proteins has been detected (28, 29), PFY1 has not been identified as allergen. The predominant yeast allergens are in the high-molecular-weight range, with enolase appearing as a major allergen. Some proteins in the range of 10–15 kDa exhibited a weak binding capacity in IgE immunoblots. Whether these proteins can cause allergic reactions is still unknown.

Moreover, histamine release from basophils was analyzed after incubation with fruit extracts from PFY1-expressing lines as well as Lyc e 1-silenced lines. Lyc1_RNAi/PFY1 line 53 and Lyc e 1-silenced line 15 showed no detectable amounts of Lyc e 1 (Fig. 5B). Consistently, no histamine release could be detected when basophils were stimulated with fruit extracts from these 2 transgenic lines (Fig. 7B). In addition, prick-to-prick skin tests were performed with fruit samples from WT, Lyc e 1-silenced, and PFY1-expressing plants. The different responses to transgenic fruits observed are most likely due to the fact that the patients involved in our study were not monosensitized to Lyc e 1 (5, 6). Additional allergens, *e.g.*, Lyc e 2, Lyc e 3, and yet not identified tomato allergens, are likely to be involved in the different skin reactivities. Therefore, single allergen suppression will not be sufficient to reduce the overall allergenicity of foods expressing multiple allergens. However, combining multiple allergen-reduced traits by crossing may ultimately lead to hypoallergenic food.

In summary, we could restore the cellular function of PFN in Lyc e 1-deficient tomato plants by simultaneous expression of yeast-derived PFN. Although PFY1 probably possesses no allergenic properties, a more detailed safety evaluation according to the weight-of-evidence approach described by the Codex Alimentarius Commission Guidelines for Allergenicity Assessment (30) will be mandatory (31). In conclusion, our proof-of-concept study presented here provides a strategy to enable transgenic reduction or elimination of functionally important allergens from fruits and vegetables while maintaining biological viability by means of complementation with nonallergenic orthologous proteins. FJ

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