

Design of tomato fruits with reduced allergenicity by dsRNAi-mediated inhibition of ns-LTP (Lyc e 3) expression

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Summary

Plant genetic engineering has the potential to introduce new allergenic proteins into foods but, at the same time, it can be used to remove established allergens. Here, we report the molecular characterization of Lyc e 3, a new tomato (*Lycopersicon esculentum*) allergen, and the efficient down-regulation of its expression in transgenic tomato plants. Following the identification of an immunoglobulin E (IgE)-binding 9-kDa polypeptide in tomato peel, designated Lyc e 3, its partial amino acid sequence was determined by N-terminal protein sequencing. Sequence comparison revealed that Lyc e 3 encodes a nonspecific lipid transfer protein (ns-LTP). In plants, ns-LTPs are encoded by large gene families which differ in primary amino acid sequence, expression and proposed cellular function. To identify Lyc e 3 encoding complementary DNAs (cDNAs), public tomato expressed sequence tag (EST) databases were screened for ns-LTP sequences. Following this strategy, two cDNAs, LTPG1 and LTPG2, with high homology to the N-terminal sequence of Lyc e 3, were identified. Ectopic expression of LTPG1 and LTPG2 in *Escherichia coli*, followed by immunoblotting, verified their IgE reactivity. Subsequently, transgenic tomato plants constitutively expressing LTPG1- or LTPG2-specific double-stranded RNA interference (dsRNAi) constructs were created and tested for the suppression of Lyc e 3 accumulation. Efficient silencing of Lyc e 3 was documented by Northern and Western blotting. In both cases, Lyc e 3 accumulation was decreased to levels below the detection limit (less than 0.5% of the wild-type protein). The allergenic potential of Lyc e 3-deficient tomato fruits was tested by measuring histamine release from sensitized human basophils stimulated with transgenic and parental lines. These assays revealed a strong (10- to 100-fold) decrease in histamine release of human basophils challenged with transgenic fruit extracts when compared with control extracts. These results demonstrate the feasibility of creating low allergenic tomato fruits by means of dsRNAi inhibition.

Keywords: allergens, dsRNAi, Lyc e 3, ns-LTP, transgenic tomato.

Introduction

In industrialized countries, food allergies are a relevant nutritional problem for humans, as any food that contains proteins has the potential to elicit an allergic reaction in a subgroup of the population. To date, more than 300 plant proteins have been shown to be able to induce immunoglobulin

E (IgE)-mediated (type I) allergic reactions, which are the most common allergic reactions in humans (IUIS Allergen Nomenclature Subcommittee, <http://www.allergen.org>). Although the prevalence of food allergy is not accurately known, it is believed that 1%–2% of the adult population and up to 8% of children suffer from food allergies. If allergies to fruits and vegetables are taken into consideration, higher prevalences

have been estimated in adults (Vieths *et al.*, 2002). Avoidance of the food is, in most cases, the only treatment available to date, thus limiting dietary choices and the quality of life of food-allergic individuals. The relevance of this problem is further increased as concomitant allergies to multiple plant-derived foods are common, in particular to fruits and vegetables (Vieths *et al.*, 2002). Plant genetic engineering has the potential to remove or reduce established allergens. This may be achieved by specifically removing IgE-binding sites, as demonstrated for Lol p 5 from ryegrass (Swoboda *et al.*, 2002), Cry j 1 from Japanese cedar (Usui *et al.*, 2003) and the food allergen Pru av 1 from cherry (Neudecker *et al.*, 2003; Wiche *et al.*, 2005), or by efficient and stable down-regulation of gene expression, as shown for Gly m Bd 30 k from soybean, 14–16-kDa protein from rice, Mal d 1 from apple and Lol p 5 (Tada *et al.*, 1996; Bhalla *et al.*, 2001; Herman *et al.*, 2003; Gilissen *et al.*, 2005). Gene silencing can be achieved by several methods, including RNA interference (RNAi), which has been shown to be most efficient, leading to almost 100% inhibition of endogenous transcript accumulation (Smith *et al.*, 2000). Although promising, plant biotechnology has not been used widely to decrease the allergenic potential of plant-derived food. Reasons for this may be the occurrence of multiple allergenic proteins and/or the possible insufficient degree of silencing. Furthermore, allergenic proteins may fulfil essential cellular functions and thus may be indispensable in plant cells. These limitations could be overcome by simultaneous silencing of several proteins, the creation of stable mutants and complementation of silenced or mutated plants using hypoallergenic variants of the selected allergenic protein. To investigate the potential of biotechnological approaches for the creation of hypoallergenic food, we have chosen tomatoes as a model system.

Tomato (*Solanum lycopersicum* or *Lycopersicon esculentum*) belongs to the Solanaceae family, native to South and Central America. After only a few hundred years in European culture, the tomato has become a major constituent of diets in many countries. From pasta sauce to pizza, tomatoes are a daily staple in Italy and many other countries, and have become one of the world's most important vegetables. They supply one of the most powerful, naturally occurring antioxidants, lycopene, and may be responsible for the low incidence of prostate cancer amongst Italian men. Therefore, tomato consumption is highly recommended; however, tomato fruits contain a number of proteins with high allergenic potential. The prevalence of tomato allergy ranges from 1.5% (Jäger and Wüthrich, 2002) to 20% (Ortolani *et al.*, 1988, 1989) in different populations of food-allergic patients, indicating that tomato is a relevant allergenic food in selected populations.

The most comprehensive study on tomato allergens was performed by Foetisch *et al.* (2001), who investigated a panel of 32 tomato-allergic patients. Two-dimensional immunoblotting combined with N-terminal microsequencing identified β -fructofuranosidase, polygalacturonase and pectinesterase as IgE-binding components in tomato extract. These data were confirmed in parallel by Kondo *et al.* (2001) in a study comprising 11 subjects with oral allergy syndrome to tomatoes. Moreover, these authors reported superoxide dismutase as a putative allergen in tomato. By the inhibition of IgE binding with the purified recombinant birch pollen profilin, Foetisch *et al.* (2001) provided strong evidence for the regulatory protein profilin being an important allergen in tomato. Later, tomato profilin was cloned and expressed in *Escherichia coli* by two groups, and was the first officially recognized allergen in tomato, Lyc e 1 (Willeroider *et al.*, 2003; Westphal *et al.*, 2004).

Later, β -fructofuranosidase was denominated as allergen Lyc e 2, and was shown to be a glycoprotein carrying complex N-glycans containing xylose and fucose residues. In accordance with preliminary data by Foetisch *et al.* (2001), molecular characterization of Lyc e 2 revealed that the glycan structures were important for the allergenicity of the polypeptide (Westphal *et al.*, 2003).

Evidence for the lipid transfer protein (LTP) Lyc e 3 representing an allergen in tomato resulted from the screening of a number of plant-derived foods for cross-reactivity with IgE antibodies to Rosaceae LTPs (Asero *et al.*, 2000) and from enzyme allergosorbent (EAST) inhibition assays (Foetisch *et al.*, 2001). In fruit extracts, a 9-kDa protein band cross-reacted with anti-Rosaceae LTP IgE. Furthermore, cherry LTP was able to inhibit IgE binding to tomato extracts. Based on homology, le16 (Plant *et al.*, 1991), a complementary DNA (cDNA) clone encoding a drought- and abscisic acid-inducible nonspecific LTP (ns-LTP), was suggested to encode Lyc e 3; however, no direct proof for the identity between le16 and Lyc e 3 has been provided. ns-LTPs belong to a large family of small hydrophilic proteins that can enhance nonspecific intermembrane lipid transfer. Recently, a function of ns-LTPs from *Arabidopsis* as calmodulin-binding proteins has been discussed (Wang *et al.*, 2005).

Allergy to a low-molecular-mass allergen in peach (~10 kDa) was first described in 1992 (Leonart *et al.*, 1992), and the allergen later turned out to represent an ns-LTP, designated Pru p 3 (Pastorello *et al.*, 1999). To date, ns-LTPs have been identified as allergens in fruits of the Rosaceae subfamilies of the Prunoideae (e.g. peach, cherry, apricot and plum) and Pomoidaceae (e.g. apple). As a result of their ubiquitous distribution in food and non-food sources, ns-LTPs have been classified as pan-allergens (Asero *et al.*, 2000; Ballmer-Weber,

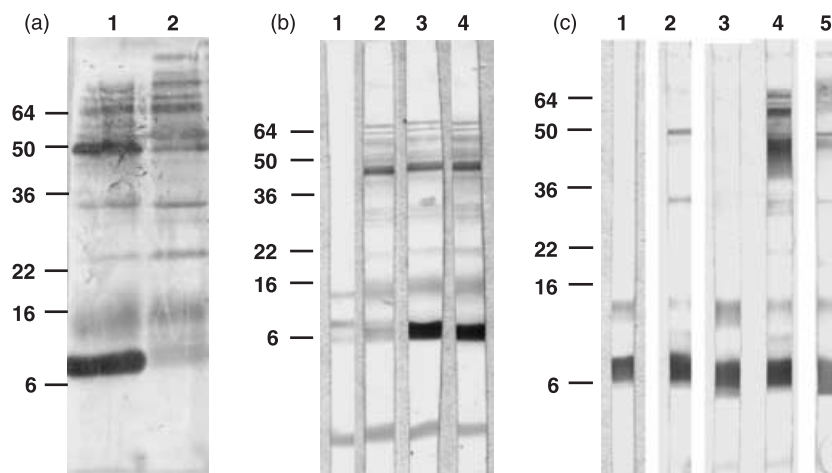


Figure 1 Identification of a nonspecific lipid transfer protein (ns-LTP) as a new tomato allergen. (a) Immunoglobulin E (IgE) binding to the 9-kDa protein in tomato extracts with serum of a tomato-allergic patient (PEI131). Molecular weights of the marker proteins (kDa) are indicated on the left. Lane 1: tomato peel extract. Lane 2: tomato flesh extract. (b) Immunoblot inhibition of IgE reactivity with serum (PEI131). Molecular weight standards (kDa) are indicated on the left. Lane 1: non-allergic control. Lane 2: serum pre-incubated with cherry ns-LTP Pru av 3 (20 µg). Lane 3: serum pre-incubated with bovine serum albumin (BSA) (20 µg). Lane 4: serum without inhibitor. (c) Comparison of IgE binding to the 9-kDa protein from tomato peel extracts with sera from five representative tomato-allergic patients with sensitization to Lyc e 3 (lanes 1–5, PVS2934, GI-2, CS-1, CS-2, CS-5). Twenty micrograms of total tomato peel protein extract were loaded in each lane.

2002; van Ree, 2002). They belong to a multigene family of structurally highly conserved cysteine-rich proteins that are also present in non-Rosaceae vegetable foods, including Solanaceae (e.g. pepper, eggplant, potato and tomato) (Asero *et al.*, 2004) and pollen. In contrast with pollen-related allergens in fruits, nuts and vegetables, which mainly cause relatively mild symptoms, there is increasing evidence that ns-LTPs are capable of eliciting severe, systemic allergic reactions in a relatively large proportion of sensitized patients (Pastorello *et al.*, 2002; San Miguel-Moncin *et al.*, 2003; Pastorello and Robino, 2004; Schocker *et al.*, 2004). For this reason, we selected ns-LTPs for our model study on allergen suppression.

In this paper, we provide, for the first time, direct evidence that two cDNAs, designated LTPG1 and LTPG2, encoding tomato ns-LTPs correspond to Lyc e 3. Furthermore, silencing LTPG1 and LTPG2 by double-stranded RNA interference (dsRNAi) led to a strong decrease in Lyc e 3 accumulation and hence to allergen-reduced tomato fruits. A correlation between a decreased potential to elicit type I allergic reactions and decreased Lyc e 3 accumulation was demonstrated by the decrease in histamine release of human basophils challenged with transgenic tomato fruit extracts.

Results

Identification of an ns-LTP as a new tomato allergen

Initially, the IgE reactivity pattern of tomato fruit extracts was characterized by sodium dodecylsulphate-polyacrylamide

gel electrophoresis (SDS-PAGE) and immunoblotting. These studies revealed that IgE from the sera of patients with type I allergy to tomato recognized a 9-kDa protein. Interestingly, this IgE-binding protein was highly enriched in tomato peel extracts relative to protein extracts from flesh (Figure 1a). The presence of a low-molecular-weight allergen in the peel of fruit has previously been described for peach and apple, and the allergen was identified as ns-LTP (Fernandez-Rivas and Cuevas, 1999). To prove the presumed identity of this band, serum from allergic patients with IgE against the 9-kDa protein was pre-incubated with purified recombinant ns-LTP from cherry, Pru av 3 (Scheurer *et al.*, 2001), resulting in an almost complete inhibition of IgE binding to the tomato protein, whereas no inhibition was observed with bovine serum albumin (BSA). No IgE binding was observed with serum from a non-allergic patient (Figure 1b). In total, 35 patients with a suggestive case history of immediate-type allergic reactions after ingestion of tomato were recruited in Spain. Sera from 10 of these patients (29%) showed IgE binding to the 9-kDa protein in tomato peel extract, indicating that it was a minor allergen in tomato. The IgE-binding pattern of five representative sera is shown in Figure 1c.

N-terminal amino acid sequencing and cloning of ns-LTP isoforms from tomato

To further confirm the identity of the allergen and to generate information on its primary structure, tomato peel extract was separated by one-dimensional SDS-PAGE. Following

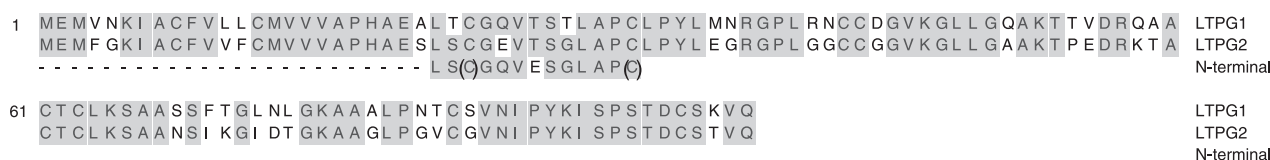


Figure 2 Alignment of the deduced amino acid sequences of LTPG1 and LTPG2 with the N-terminal amino acid sequence obtained by sequencing the allergic nonspecific lipid transfer protein (ns-LTP) purified from tomato peel.

electrophoresis, polypeptides were blotted on to polyvinylidene difluoride membranes and a protein band with a molecular mass of 9 kDa was excised. The N-terminal sequence of the 9-kDa protein band was determined by Edman degradation and found to be: LS(C) GQVESGLAP(C).

Searching public databases (i.e. dbEST) for tomato ns-LTP cDNA sequences revealed 18 ns-LTP expressed sequence tag (EST) sequences. Evaluation of these sequences allowed the assembly of two contigs, designated LTPG1 and LTPG2, covering the entire ns-LTP coding region. One of these contigs (LTPG2) was highly similar to the previously isolated ns-LTP cDNA le16. Comparing the deduced amino acid sequences of LTPG1 and LTPG2 with the N-terminal amino acid sequence of Lyc e 3 showed that both clones were highly homologous to the predicted tomato allergenic ns-LTP isoform (Figure 2). Based on the available nucleotide sequences, polymerase chain reaction (PCR) primers were designed (see 'Experimental procedures') and used to amplify and clone the entire coding regions of LTPG1 and LTPG2 from *L. esculentum* var. Microtom. Both sequences have been deposited in the EMBL Nucleotide Sequence Database (Acc. No. AM051295; AM051296).

LTPG1 and LTPG2 expressed in *E. coli* bind IgE from tomato-allergic patients

To verify that LTPG1 and LTPG2 encode allergenic Lyc e 3 ns-LTP isoforms, both proteins were expressed in *E. coli* and purified. To this end, LTPG1 and LTPG2 cDNAs, coding for the mature proteins, were amplified by PCR and cloned in frame into the pET100D expression vector. Recombinant proteins were purified under native conditions using metal chelate affinity chromatography. The purified proteins were subjected to SDS-PAGE (0.5 µg/cm) and blotted on to nitrocellulose membrane. Subsequently, the nitrocellulose membrane was cut into strips, and each strip was tested for reactivity with different antibody solutions. As shown in Figure 3, recombinant LTPG1 and LTPG2 clearly bind IgE from the sera of two tomato-allergic patients (PEI131 and PVS2934). Interestingly, LTPG2 reacted more strongly to serum PVS2934 than serum PEI131. This different response was not found for LTPG1, which was recognized by both sera to the same extent, indic-

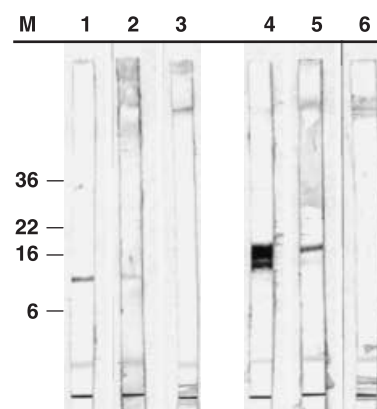


Figure 3 Immunoglobulin E (IgE)-binding analysis with sera from two different tomato-allergic patients to recombinant LTPG1 (lanes 1–3) and LTPG2 (lanes 4–6). M, molecular weight marker. Lanes 1 and 4: serum PVS2934. Lanes 2 and 5: serum PEI131. Lanes 3 and 6: buffer control.

ating that the two different sera distinguish between the two LTP isoforms. In addition, differential immunogenicity of both isoforms was confirmed by a cross-reactive antibody raised against the ns-LTP from hazelnut (data not shown). Equal loading was verified by probing the blots with an anti-histidine tag IgG antibody (data not shown). On the basis of these results, LTPG1 and LTPG2 encode two new tomato allergens referred to as Lyc e 3.01 and Lyc e 3.02, respectively.

Lyc e 3 has been included in the official list of allergens of the IUIS Allergen Nomenclature Subcommittee.

Generation of Lyc e 3-reduced tomato fruits by dsRNAi inhibition of LTPG1 and LTPG2 expression

To generate tomato fruits with reduced Lyc e 3 expression, a dsRNAi inhibition approach was applied. To this end, the entire coding region of LTPG1 or LTPG2 was cloned in sense and antisense orientation, separated by the first intron of the potato GA20 oxidase gene, into the vector pUC-RNAi (Chen *et al.*, 2003). Subsequently, the hairpin cassettes were inserted into the vector pBinAR, between the cauliflower mosaic virus (CaMV) 35S promoter and the octopine synthase (*ocs*) terminator, yielding pBin-LTPG1_RNAi and pBin-LTPG2_RNAi, respectively (Figure 4). The binary RNAi constructs were

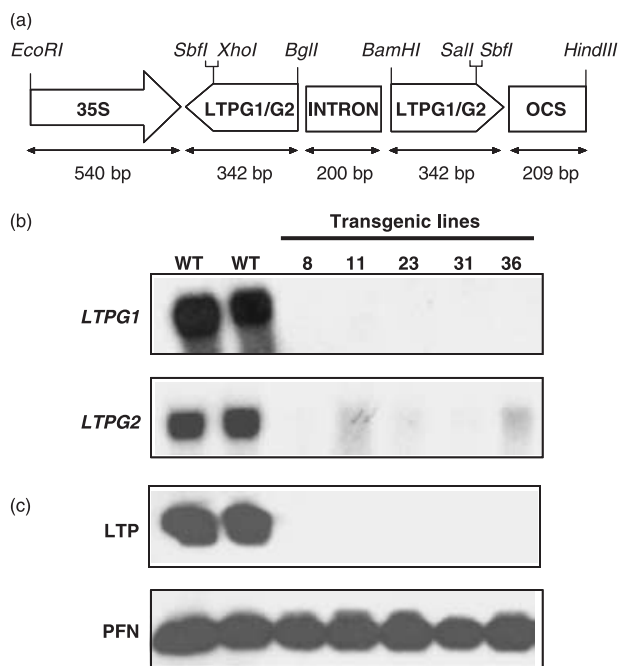


Figure 4 Expression analysis of lipid transfer protein (LTP) in transgenic tomato plants. (a) LTP_RNAi constructs used to generate transgenic plants. The complete LTPG1 or LTPG2 coding region was introduced in sense and antisense orientation between the constitutive cauliflower mosaic virus (CaMV) 35S promoter and the octopine synthase (ocs) terminator. Sense and antisense fragments were separated by an intron derived from the GA-20 oxidase gene from *Solanum tuberosum*. (b) Northern blot analysis of pBin-LTPG1_RNAi transgenic plants. Total RNA was isolated from tomato peel of five independent transgenic lines (8, 11, 23, 31, 36) and Microtom wild-type plants (lanes 1, 2). Twenty micrograms of total RNA were loaded on each lane and hybridized with labelled [^{32} P] cDNA from LTPG1 or LTPG2 as indicated. (c) Western blot analysis of transgenic plants. Protein extracts were prepared from peel of tomato fruits from five different pBin-LTPG1_RNAi transgenic lines (8, 11, 23, 31, 36) and Microtom wild-type plants. Ten micrograms of total peel protein were applied to each lane. Western blot was performed using a rabbit-raised polyclonal anti Cor a 8 LTP antiserum (dilution 1 : 10 000) and a goat-raised anti-rabbit IgG antibody (dilution 1 : 20 000). LTP migrated at approximately 10 kDa. As loading control, membranes were probed for the presence of profilin using a rabbit-raised polyclonal anti-ragweed profilin antiserum (dilution 1 : 10 000).

transformed into tomato by *Agrobacterium*-mediated gene transfer in separate experiments for each isoform, and 50 kanamycin-resistant tomato plants each were generated and analysed.

As Lyc e 3 was most abundant in the peel of tomato fruits, transgenic plants were screened for the loss of Lyc e 3 RNA and protein in peel extracts. Following mechanical separation of the peel from tomato fruits, protein extracts were separated by SDS-PAGE and blotted on to nitrocellulose membranes. For immunodetection of Lyc e 3, rabbit polyclonal antibodies directed against LTP from hazelnut (Cor a 8) were used. As shown in Figure 4c, the Cor a 8 antibody cross-reacted

strongly with Lyc e 3 in protein extracts from wild-type peel. This protein band was not detected by the antiserum in peel extracts from five independent pBin-LTPG1_RNAi transgenic lines (transgenic lines 8, 11, 23, 31 and 36). As loading control, the same blot was incubated with polyclonal antiserum against profilin. As shown in Figure 4c, the profilin content in wild-type and transgenic protein extracts was indistinguishable, indicating that similar amounts of proteins had been loaded. In order to confirm these results at the messenger RNA (mRNA) level, total RNA from tomato peel was isolated, separated by formaldehyde-containing agarose gel electrophoresis and blotted on to nylon membranes. The accumulation of Lyc e 3 mRNA was probed by hybridizing the RNA blot with radio-labelled cDNAs from LTPG1 and LTPG2. In the wild-type, both probes detected substantial amounts of Lyc e 3 mRNA (Figure 4b). In RNA extracts from pBin-LTPG1_RNAi transgenic plants, however, LTPG1 and LTPG2 gave different results. Although no LTPG1 hybridizing signal could be detected, LTPG2 detected some remaining RNAs in lines 11, 23 and 36. Thus, both isoforms were obviously efficiently silenced by the pBin-LTPG1_RNAi construct. Although LTPG1 and LTPG2 are only 76.5% identical at the nucleotide level, five stretches with identities of more than 15 nucleotides can be found within the coding region. Based on these stretches, small single-stranded RNAs, able to direct the RNA-induced silencing complex (RISC) to both target sequences, can be generated.

The same Western and Northern analysis was performed with pBin-LTPG2_RNAi transgenic lines and similar results were obtained. Five lines (8, 26, 37, 45 and 49) with no detectable LTPG1 and LTPG2 RNA and protein were selected for further investigations (data not shown).

To further quantify the efficiency of dsRNAi inhibition, the detection limit of the anti-Cor a 8 polyclonal antiserum was tested. To this end, wild-type extracts were diluted from 20 μ g to 0.1 μ g of total protein and Lyc e 3 was visualized by Western blotting. In all dilutions, Lyc e 3 protein could be detected (data not shown). However, no remaining Lyc e 3 protein could be detected in 20 μ g of total protein extracts from transgenic fruits (data not shown). Therefore, we conclude that Lyc e 3 accumulation in transgenic fruits has been decreased below 0.5% of the wild-type levels.

Transgenic tomato fruits show decreased allergenic potential in histamine release assays

The ability to induce histamine release from human basophils was investigated in order to prove, in a functional assay, whether the decrease in ns-LTP expression is translated into decreased allergenic potential of the transgenic plants. The

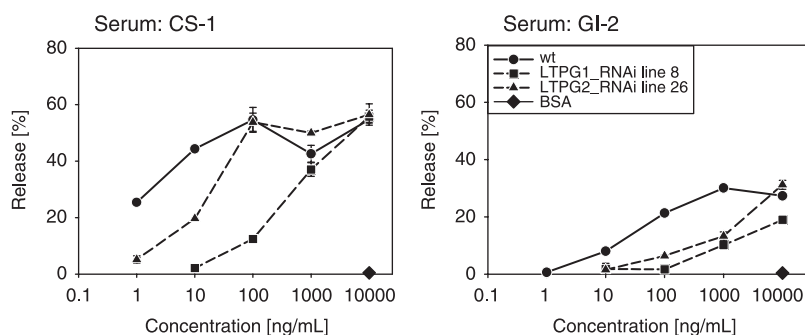


Figure 5 Induction of histamine release from stripped human basophils. Human basophil cells from non-allergic donors were passively sensitized with sera from tomato-allergic patients: sera CS-1 and GI-2. Subsequently, they were stimulated with diluted tomato fruit extracts from wild-type (cv. Microtom) plants and from the transgenic lines pBin-LTPG1_RNAi (line 8) and pBin-LTPG2_RNAi (line 26). The concentrations of extracts varied from 1 to 10 000 ng/mL. Histamine release was quantified as described in 'Experimental procedures'. Standard deviations are shown for each measurement. BSA, bovine serum albumin.

histamine release assay was performed with stripped basophils from non-allergic donors, passively sensitized with serum from tomato-allergic patients. All tomato extracts were dialysed extensively to remove endogenous histamine. For histamine release experiments, pBin-LTPG1_RNAi line 8 and pBin-LTPG2_RNAi line 26 were chosen and compared with untransformed controls. In both transgenic lines, Lyc e 3 accumulation was decreased below the detection limit. The biological potency of the RNAi plants to cross-link specific IgE bound to high-affinity receptors on effector cells was determined with two sera (Figure 5). Wild-type control extracts and extracts from both pBin-LTPG1_RNAi line 8 and pBin-LTPG2_RNAi line 26 released histamine from the passively sensitized basophils in a dose-dependent manner. However, with regard to the biological potency, a 10- to 100-fold higher concentration of protein extracts from transgenic lines was needed to induce the same histamine release response as from wild-type extracts.

Discussion

The tomato allergen Lyc e 3 belongs to the superfamily of ns-LTPs which are encoded by multigene families. These gene families were discovered 30 years ago and encode proteins of 9–11 kDa in size. BLAST search at <http://www.arabidopsis.org> identified more than 30 putative ns-LTP genes in the genome of *Arabidopsis thaliana* (Chardin *et al.*, 2003; Marion *et al.*, 2004). ns-LTPs belong to the pathogenesis-related protein (PR-14) family (van Loon and van Strien, 1999); however, their precise function is not well understood. Ectopic expression of LTP2 from barley in transgenic *Arabidopsis* plants enhanced the tolerance to bacterial pathogens (Molina and Garcia-Olmedo, 1997). Furthermore, mutation of the *DIR-1* gene, encoding a putative apoplastic ns-LTP, compromised systemic acquired resistance of *Pseudomonas syringae*-challenged *Arabidopsis* plants (Maldonado *et al.*, 2002). In tomato, ns-LTP isoforms are salt- and drought-responsive, which indicates a possible role of ns-LTPs in the repair of

stress-induced membrane damage (Plant *et al.*, 1991; Torres-Schumann *et al.*, 1992; Trevino and O'Connell, 1998). A role of ns-LTP in membrane reorganization is further supported by the identification of germination-specific ns-LTP cDNA sequences in *Brassica napus* (Soufleri *et al.*, 1996). The mode of action of ns-LTPs is largely unknown; however, the recent finding that members of the ns-LTP family may act as calmodulin-binding proteins indicates a possible link between the Ca^{2+} /calmodulin system and lipid metabolism (Liu *et al.*, 2001; Wang *et al.*, 2005). In addition to their possible roles in plant defence and metabolism, members of the ns-LTP family are potent allergens found in food and non-food sources. As a result of their wide distribution and high degree of IgE cross-reactivity, they have been classified as pan-allergens (Asero *et al.*, 2000; Ballmer-Weber, 2002).

ns-LTPs are common in various kinds of pollen and have been shown to be minor allergens in most cases, with the exception of *Parietaria judiaca* pollen, in which proteins with homology to ns-LTPs represent the major pollen allergens. By contrast, ns-LTPs were identified as major allergens in many foods, in particular in fruits of the Rosaceae family, but also in hazelnut (Schocker *et al.*, 2004), maize (Pastorello *et al.*, 2000), grape (Pastorello *et al.*, 2003) lettuce (San Miguel-Moncín *et al.*, 2003) and many other plant-derived foods (Pastorello and Robino, 2004), when patients from the Mediterranean area were studied. The fact that allergic reactions to ns-LTP tend to be severe may be related to its extreme stability to digestion (Asero *et al.*, 2000; Scheurer *et al.*, 2004) and food processing, resulting in ns-LTP-containing heat-processed foods, such as fruit juice or jam (Scheurer *et al.*, 2004). This is in strong contrast with most other fruit allergens which are rapidly inactivated by thermal processing.

The IgE-binding data presented here confirm previous findings (Asero *et al.*, 2000; Foetisch *et al.*, 2001), pointing towards the existence of an allergenic ns-LTP in tomato as a minor allergen. According to the rules of the IUIS Allergen Nomenclature Subcommittee, an allergen is defined as a minor allergen if the prevalence of IgE binding to a (glyco)protein

is between 5% and 50% within a patient population allergic to the respective source material, and at least five patients are sensitized to this protein. The assignment of Lyc e 3 as a minor allergen contrasts with the importance of ns-LTP as a major food allergen in plant food allergy in Spain and Italy. This apparent discrepancy may be related to the fact that many of our tomato-allergic patients had concomitant sensitization to grass pollen and other pollen species, which contain allergenic profilins, leading to a cross-reactive sensitization to tomato profilin (Willeroider *et al.*, 2003; Westphal *et al.*, 2004) in the majority of our patients. The importance of profilin in tomato allergy and the potential to further reduce the allergenicity of tomatoes by suppression of profilin will be addressed in a separate study.

Although le16, encoding a drought-inducible ns-LTP from tomato, has been postulated to encode Lyc e 3, this has not been demonstrated experimentally.

In this study, we identified and characterized two coding sequences, LTPG1 and LTPG2, and demonstrated that they encode Lyc e 3.01 and Lyc e 3.02, two isoforms of the same allergen. The cDNAs were isolated from fruits of *L. esculentum* var. Microtom. The coding regions of the two cDNAs were 76.5% identical at the nucleotide level. The 345-base pair (bp) open reading frames encode precursor polypeptides of 114 amino acid residues, including a 24-amino acid N-terminal signal peptide. The mature polypeptides were predicted to comprise 90-amino acid residues with calculated molecular weights of 9.307 kDa (LTPG1) and 8.855 kDa (LTPG2) and pI values of 8.68 (LTPG1) and 8.11 (LTPG2). At the amino acid level, the two mature proteins were 73.3% identical. Both genes were highly expressed in the peel of tomato fruits, leading to the accumulation of Lyc e 3 in this tissue. Enrichment of allergenic ns-LTP in peel has also been shown for peach (Pru p 3) and apple (Mal d 3) (Fernandez-Rivas and Cuevas, 1999; Pastorello and Robino, 1999; Carnes *et al.*, 2002).

Sera from tomato-allergic patients were tested for IgE reactivity with recombinant LTPG1 and LTPG2 proteins produced in *E. coli*. Both polypeptides bound IgE from two tomato-allergic patients. As shown for the isoforms of allergens from other allergenic foods, such as cherry (Reuter *et al.*, 2005) or carrot (Ballmer-Weber *et al.*, 2005), the IgE-binding capacities of LTPG1 and LTPG2 were found to be different. Although LTPG1 was moderately recognized by IgEs from both sera, LTPG2 strongly reacted with serum PVS2934, but only moderately with serum PEI131. However, studies with a larger number of patients are required to confirm this finding. Moreover, these differences may indicate differences between the IgE epitopes of LTPG1 and LTPG2. In the case of Lyc e 3, IgE-binding sites have not been determined; however, IgE-

binding sites have been mapped on Pru p 3, coding for an ns-LTP with high homology to Lyc e 3. Using peptide scanning, Garcia-Casado *et al.* (2003) determined three regions [amino acids 11–20 (P1), 31–40 (P2) and 71–80 (P3)] on the Pru p 3 polypeptide responsible for IgE binding. At the amino acid level, the identity between Pru p 3 and Lyc e 3 is 50% (LTPG1) and 53.3% (LTPG2). With respect to the major IgE-binding sites, P1 and P3 are conserved between all three ns-LTP sequences. Although P1 is identical between LTPG1 and LTPG2, P3 shows some sequence variation which might be responsible for the differences observed in IgE binding.

To unequivocally demonstrate that LTPG1 and LTPG2 encode Lyc e 3.01 and Lyc e 3.02, respectively, transgenic tomato plants with decreased expression of both genes were created. Silencing was achieved by RNAi in transgenic tomato plants. As there is sufficient sequence similarity between LTPG1 and LTPG2, silencing of both genes was achieved by introducing the complete coding regions of either gene in sense and antisense orientation between the CaMV 35S promoter and the ocs terminator. To enhance the efficiency of silencing, sense and antisense fragments were separated by an intron-containing spacer. Northern and Western blot analysis verified the inhibition of LTPG1 and LTPG2 expression. Quantitative Western blot analysis revealed that the accumulation of Lyc e 3 in the peel of transgenic tomato fruits was decreased below 0.5% of the wild-type levels.

The effect of gene silencing on the potential allergenicity of transgenic tomato plants was tested by histamine release from human basophils challenged with transgenic and wild-type fruits. Histamine release was decreased when basophils were inoculated with transgenic fruits. The failure to obtain tomato fruit extracts unable to induce histamine release may be explained by: (i) the residual accumulation of Lyc e 3; and/or (ii) the presence of additional polypeptides to which the patients were sensitized. As shown in Figure 1, other IgE-binding bands were weakly detected in tomato extract, which could account for one-tenth to one-hundredth of the allergenic potency of Lyc e 3. In the patients shown in Figure 1, the molecular weight of the additional allergen (approximately 50 kDa) may correspond, for example, to the glycosylated tomato allergen, Lyc e 2 (Westphal *et al.*, 2003). One important characteristic of immune responses to food proteins in allergy is that multiple allergenic proteins exist in one food source, and subgroups of patients are concomitantly sensitized to more than one allergen in a given food. For peanut, for example, eight different allergens are known, and cow's milk contains five allergens (<http://www.allergen.org/>). Thus, a 90%–99% decrease in allergenicity, which was achieved in this study, is considered to have a strong effect

for certain patients. However, the beneficial effect of Lyc e 3-suppressed tomatoes for tomato-allergic patients must be tested in clinical trials by food challenge tests. The implementation of provocation tests (e.g. skin prick testing and oral challenge tests) is mandatory to verify the reduced allergenicity of transgenic lines in a representative patient population. However, the aim of the present study was to provide proof of concept and to show the potential of RNAi silencing as a powerful tool for the creation of low allergenic food.

Attempts to decrease the allergenic potential of food sources have been published for rice, soybean, apple and peanut. In rice, decreased accumulation of a 14–16-kDa allergenic protein has been reported. However, the decrease was found to be only moderate, with approximately 20% of the allergenic protein remaining (Tada *et al.*, 1996). In soybean, both genetic and reversed genetic approaches have been followed to reduce the accumulation of major allergens. Using mutant soybean, Samoto *et al.* (1998) selected soybean plants with substantial reductions in the accumulation of three putative major allergens (Gly m Bd 30K, Gly m Bd 28K and the α -subunit of conglycinin). Following a co-suppression approach, Herman *et al.* (2003) inhibited the expression of Gly m Bd 30K in transgenic soybean plants. In both cases, functional tests, such as triggering of effector cells to demonstrate the decrease in potential allergenicity, were not performed. RNAi has been used to decrease the accumulation of Mal d 1 in transgenic apple (Gilissen *et al.*, 2005). As a result of the long regeneration time of apple, only leaves were tested for decreased allergen accumulation. Based on skin prick tests and immunoblotting, Mal d 1 expression in leaves was substantially decreased. However, these results need to be confirmed in fruits of transgenic apple trees to support the feasibility of producing hypoallergenic Mal d 1-deficient apples by gene silencing. Without providing any experimental evidence, Dodo *et al.* (2005) reported the inhibition of Ara h 2, the most potent peanut allergenic protein.

In addition to molecular approaches to inhibit the accumulation of food allergens, silencing of a major pollen allergen, Lol p 5, has been reported for ryegrass (Bhalla *et al.*, 2001). As discussed above, a small number of transgenic approaches to reduce the allergenic potential of food and non-food sources have been reported. The application of RNAi has the potential to allow the design of hypoallergenic transgenic plants. In comparison with traditional breeding strategies, it allows the simultaneous silencing of multigene families (homology-based silencing of related genes) and of multiple target genes (chimeric RNAi) in a cell- and tissue-specific manner. In combination with specific promoter elements, silencing can be restricted to the target tissues, thereby

minimizing negative effects due to loss of protein function. Theoretical down-sides of RNAi are that null mutants cannot be obtained and that RNAi constructs may not be stably inherited in following generations, which must be proven in long-term studies. Moreover, the silencing efficiency may be influenced by environmental factors, such as low temperatures and viral infections. Using *Nicotiana benthamiana* as a model system, Szittyá *et al.* (2003) demonstrated that siRNA accumulation and efficient silencing of viral and transgenes were negatively affected by low temperatures. In addition, several lines of evidence suggest that RNA silencing is an antiviral defence mechanism. Therefore, it is not surprising that many plant viruses encode suppressors of RNA silencing (for a review, see Roth *et al.*, 2004), which have the potential to jeopardize RNAi approaches in transgenic plants. If true, alternative strategies to select hypoallergenic plants must be followed. One example is TILLING (targeting-induced local lesion in genomics), which provides the possibility to obtain an allelic series of induced point mutants in any gene of interest (Slade and Knauf, 2005). Thus, TILLING would allow null mutants to be obtained and, furthermore, represents a non-genetically modified strategy to produce hypoallergenic food.

Experimental procedures

Patients' sera

Sera were taken from Spanish patients with adverse reactions after the ingestion of tomato fruits ($n = 35$). Allergy diagnosis was based on a positive case history and positive serum IgE test results. Most of the patients reported the oral allergy syndrome as a symptom after tomato ingestion. Tomato-specific IgE was determined by the CAP FEIA system (Pharmacia Diagnostics, Uppsala, Sweden) according to the manufacturer's instructions. Only patients with specific IgE to tomato (CAP > 0.35 kU/L; CAP class 1) (CAP: capsulated hydrophobic carrier polymer) were used in this study. The sera of healthy non-allergic and allergic subjects with no tomato allergy and no specific IgE to tomato were used as negative controls.

Plant material and growing conditions

Tomato plants (*L. esculentum* cv. Microtom) were grown in tissue culture under a 16-h light/8-h dark cycle on Murashige–Skoog medium containing 2% (w/v) sucrose. After the initial characterization of *in vitro*-grown plants, tomato plants were transferred to the glasshouse. Plants were cultivated in soil with 16 h supplemental light followed by 8 h of darkness. The temperature regime followed by the day/night cycle was 25 °C/20 °C.

Preparation of tomato protein extracts

For allergen preparations, protein extracts from tomato peel and tomato pulp were prepared using a low-temperature method. Fresh fruits were separated into peel and pulp. Aliquots were frozen in liquid nitrogen and powdered using a Grindomax 200M mill (Retsch GmbH & Co KG, Haan, Germany). Subsequently, the powder was homogenized in ice-cold diacetone alcohol and acetone and stored overnight at -20°C . After filtration and washing with acetone and acetone–diethylether (1 : 1), the powder was lyophilized and stored at -20°C . For extraction, the powder was re-suspended with phosphate-buffered saline (PBS) (0.15 M NaCl, 0.002 M KCl, 0.006 M Na_2HPO_4 ; pH 7.1) and centrifuged at 4°C . The supernatant was filtered, lyophilized and stored at -20°C . For immunoblotting, protein extracts from tomato peel were prepared as described in Biemelt *et al.* (2003). Protein concentrations were determined according to Bradford (1976) using the BioRad protein assay (BioRad, Munich, Germany).

IgE immunoblot and IgE immunoblot inhibition

Allergen extracts (20 μg of total protein/cm) were separated by SDS-PAGE according to Laemmli (1970) under non-reducing conditions in a Mini-Protean 3 cell (BioRad). Proteins were transferred on to 0.2- μm nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) by tank blotting for 45 min at 150 V (Mini Trans blot cell, BioRad). After blocking in 5% non-fat dry milk, 0.05% Tween 20, TBS (20 mM Tris, 500 mM NaCl) pH 7.4 for 2 h, the membranes were cut into strips and incubated with 1 : 6.7 diluted patients' sera overnight. Immunostaining of bound IgE antibodies was performed with a biotin-conjugated goat-antihuman IgE antibody (Kirkgaard & Perry Laboratories, Gaithersburg, MD, dilution 1 : 750, 1 h) and streptavidin alkaline phosphatase (dilution 1 : 3000, 0.5 h). Visualization was carried out using a BioRad Alkaline Phosphatase Conjugate Substrate Kit (BioRad).

Inhibition assays were performed to verify that the band determined as LTP was the target protein. Diluted patients' sera (1 : 6.7) were pre-incubated with 20 μg of recombinant cherry ns-LTP (Pru av 3) for 2 h, and IgE binding was detected as described above.

IgG immunoblot

Protein extracts were mixed with SDS-containing sample buffer (Laemmli, 1970) and, after heat denaturation 10 μg of total proteins was separated on 15% (v/v) SDS-containing polyacrylamide gels. Proteins were transferred on to nitrocel-

lulose membranes (Hybond C, Amersham Pharmacia Biotech, Braunschweig, Germany), blocked for 2 h in 5% skimmed milk/TBST (20 mM Tris, 500 mM NaCl, 0.1% (v/v) Tween 20) and incubated overnight at 4°C with rabbit antiserum raised against the Cor a 8 protein (diluted 1 : 20 000) or anti-ragweed profilin antiserum (diluted 1 : 10 000). Immuno-detection was performed using the ECL system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

N-terminal amino acid sequencing

A tomato peel extract (20 μg of total protein/cm) was separated by SDS-PAGE as described above and blotted on to a polyvinylidene difluoride membrane. The membrane was stained with Coomassie Brilliant Blue and the protein band at 9 kDa was cut out and analysed on an Applied Biosystems 492 Procise sequencer (Applied Biosystems, Foster City, CA, USA) in pulse-liquid mode to determine the N-terminal partial sequence of the IgE-reactive protein. All chemicals were from Applied Biosystems (Weiterstadt, Germany).

Cloning of tomato ns-LTP-encoding cDNAs

Putative EST sequences encoding tomato ns-LTPs were obtained by searching public databases. Using DNA Star software (DNASTAR, Madison, WI), retrieved sequences were aligned, and two contigs (LTPG1 and LTPG2) covering the entire ns-LTP coding region were assembled. To obtain cDNA clones corresponding to these assembled sequences, a PCR approach was followed. Peel was mechanically removed from tomato fruits of different stages (green, breaker, immature and mature ripe). Isolation of total RNA from peel was performed as described by Slater *et al.* (1985). RNA was reverse transcribed with the First Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech), following the manufacturer's instructions, using 2.5 μg of total RNA for each transcription and d (T) 25 oligonucleotides as primer. To obtain the complete sequences, the reverse transcripts were amplified using gene-specific 5' and 3' oligonucleotides which were designed on the basis of LTPG1 and LTPG2 sequences: 5'LTPG1: GGTACCTTAAGAAATGGAAAT-GGTTAACAAG; 3'LTPG1: GTCGACCTGAACCTTAGAACAGTC; 5'LTPG2: AGGTACCTAACAAATGGAAATGTTTGG; 3'LTPG2: AGTCGACCTGGACCGTTGAGCAATC.

The PCR conditions were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 20 s, 55°C for 60 s and 72°C for 90 s, with a final extension step at 72°C for 10 min. PCR products were subcloned into pCR blunt vector (Invitrogen, Karlsruhe, Germany) and subjected to sequence analysis.

Protein expression in *E. coli*

For protein expression, the coding regions of LTPG1 and LTPG2 without signal peptide were cloned into pET100D-TOPO vector (Invitrogen, Karlsruhe, Germany) containing a His tag. Coding regions were amplified from LTPG1 and LTPG2 using the following specific 5' and 3' oligonucleotides: 5'LTPG1: CACCTTAACTGTGGTCAAGTTACATC; 3'LTPG1: TTAGTCGACCTGAACCTTAGAACAGTC; 5'LTPG2: CACCGAGTCACTGAGCTGCGGCG; 3'LTPG2: TTATCGACCTGGACCGTTGAGCAATC.

PCR amplification was carried out using Pwo Polymerase (PreQ Laboratory, Erlangen, Germany) under the following conditions: 94 °C for 5 min, followed by 94 °C for 30 s, 50 °C for 90 s and 72 °C for 90 s (30 cycles) and, finally, 72 °C for 7 min. For expression, pET100D constructs were transformed in *E. coli* BL21 star (Invitrogen, Karlsruhe, Germany). Single colonies were inoculated in 10 mL of Luria–Bertani (LB) medium containing ampicillin (250 mg/L) overnight at 37 °C. On the next day, 5 mL of the cell suspension was transferred into 1 L of LB medium containing ampicillin (250 mg/L). Protein synthesis was induced with 1 mM isopropyl thio- β -D-galactoside at OD₆₀₀ = 0.6 for 4 h at 37 °C. Purification of the expressed His tag protein was performed by Ni²⁺-nitrilotriacetate (Ni-NTA) agarose chromatography following the instructions of the manufacturer (Qiagen, Hilden, Germany).

RNAi vector construction

To generate the RNAi constructs, the coding regions of LTPG1 and LTPG2 were cloned in sense and antisense orientation into the pUC-RNAi vector separated by an intron (Chen *et al.*, 2003). To this end, the 342-bp ns-LTP fragments were excised from the PCR blunt vector using the *Bam*HI/*Sal*I restriction sites and inserted in sense orientation downstream of the intron into the pUC_RNAi vector. The same fragments were inserted in antisense orientation into the *Bgl*II/*Xho*I sites of pUC-RNAi already carrying the respective sense fragments. Subsequently, the entire inserts were excised using the flanking *Sbf*I sites and were inserted into the polylinker site of the binary vector pBinAR (Hoefgen and Willmitzer, 1990) between the constitutive CaMV 35S promoter and the ocs polyadenylation signal, yielding plasmids pBin-LTPG1_RNAi and pBin-LTPG2_RNAi, respectively.

Plant transformation

The final constructs were transformed to *Agrobacterium tumefaciens* CV58C1, carrying the virulence plasmid pGV2260. Tomato cotyledon explants were transformed and regenerated as described by Ling *et al.* (1998).

RNA isolation and Northern blot analysis

Twenty micrograms of total RNA were isolated as described above, separated by electrophoresis on 1.5% formaldehyde-containing agarose gels and blotted on to nylon membranes (GeneScreen, NEN, Boston, MA, USA) by capillary blotting overnight. The membranes were pre-hybridized and hybridized at 65 °C. Radioactive labelling of cDNA fragments (LTPG1/G2, *Eco*RI digested from pCR blunt vector) was performed with α -³²P-dCTP using the High Prime Kit (Boehringer Mannheim, Germany). After intensive washing, the membranes were exposed to X-ray film (Kodak, Heidelberg, Germany) over 2–3 days at –80 °C.

Basophil histamine release

Basophil histamine release utilizing stripped and re-sensitized basophils from normal donors was performed as described previously (Westphal *et al.*, 2003). Allergens were diluted for stimulation of the cells to final concentrations of 1, 10, 100, 1000 and 10 000 ng/mL. Released histamine was quantified by a commercial enzyme immunoassay (Immunotech, Marseille, France). After subtraction of the spontaneous release of the basophils, the allergen-induced histamine release was calculated as the percentage of the total amount of histamine determined after lysis of the basophils by twofold freezing and thawing.

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