

Identification of putative new tomato allergens and differential interaction with IgEs of tomato allergic subjects

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Summary

Background Tomato became one of the world-wide most consumed vegetables, unfortunately accompanied by an increasing risk of tomato allergy affecting certain people. As tomato allergic subjects show highly variable reactions in clinical allergy tests, it is difficult to identify cultivars or differentially treated tomato plants where a significant reduction in the allergenic potential over all subjects of a cohort can be detected.

Objective This study was carried out to test the hypothesis that individual variability is based on differential reactions of single subjects to particular allergens in tomato fruits of plants with certain genetic background or cultivated under distinct conditions.

Methods Proteins were extracted from tomato fruits of the previously investigated genotypes 76R, its mycorrhizal mutant RMC, and the cultivar Counter, fertilized with different forms of nitrogen in deficit or excess. 2-D immunoblots were carried out with sera of nine tomato allergic subjects, beforehand analysed in skin prick tests.

Results In total, ten putative tomato allergens were identified in these immunoblots. No correlation was detected between individual skin prick test results and the quantity of positive reactions to putative allergens. IgEs of each subject showed reactions to nearly every identified putative allergen, but reactions were dependent on genotype and growth conditions. Among the ten putative tomato allergens, five new candidates were identified as follows: an endo- β -mannanase, a pectinacetyl esterase, a pectinesterase inhibitor, an aspartyl protease family protein and a protein of unknown function.

Conclusion and Clinical Relevance The hypothesis that high interindividual differences in allergic reactions are based on the interactions between the IgEs of allergic subjects with particular allergens has to be rejected. However, five proteins with putative clinical relevance as tomato allergens could be newly identified.

Keywords immunoblot, skin prick test, tomato allergens, tomato allergy, western blot
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Introduction

Tomato is one of the world-wide most consumed vegetables (<http://faostat.fao.org>). Raw or processed in various forms, it contains numerous health-related compounds. Among those, carotenoids are discussed to prevent, for example, cardiovascular diseases and cancer [1, 2]. Unfortunately, some consumers have to avoid tomato in their daily diet because of the risk to develop allergic reactions after consumption, ranging from oral allergic symptoms to reactions of the gastrointestinal tract and the cardiovascular system [3]. Among the

European population, approximately 2–4% of the adults are affected by food allergies [4]. In recent publications, tomato has been confirmed as one of the most prevalent allergenic vegetable [5, 6]. Thus, a market for tomato fruits with reduced allergenic potential might emerge.

Different strategies can be followed to diminish the strength of allergic reactions. One strategy is the selection of particular cultivars with reduced allergenic potential. Previously, we could show that indeed, the choice of tomato cultivar significantly influenced the allergic reaction of tomato allergic subjects [7]. This

implicates that breeding programmes, including, for example, old varieties with lower allergenic potential, may result in cultivars which have a potential of save consumption for affected consumers. Another strategy is the down-regulation of allergen encoding genes in transgenic plants. This has been successfully shown for the two tomato allergens Sola 1 1 and Sola 1 3. Fruits of plants with reduced expression of one of the two allergens showed decreased allergenicity [8, 9]. However, genetically modified food is not yet accepted by the European population, and other strategies reducing the allergenic potential of food are of high commercial interest. One strategy could be the control of environmental factors. Corresponding experiments have been carried out with different success. While the application of the plant hormones ethylene and salicylic acid increased the allergic reactions to respective tomato fruits [10], different nitrogen fertilizers or the colonization of the roots with symbiotic mycorrhizal fungi had no significant impact on the overall allergenicity [11, 12]. These two latter studies performed by our group revealed, however, high individual allergic reactions of investigated tomato allergic subjects. We hypothesized that this high variation is due to individual reactions to certain allergens, differentially expressed in fruits from different genotypes, produced under particular environmental conditions. Therefore, we investigated tomatoes firstly from our previous study, where the allergenic potential of the two genotypes 76R and its mycorrhizal mutant RMC has been compared [11]. Secondly, we investigated tomatoes from another previous study with plants of the cultivar Counter, fertilized with NO₃ or organic nitrogen each in deficit or excess [12]. We analysed the allergen profile via two-dimensional (2-D) immunoblots with the sera of nine tomato allergic subjects. Therewith, we could identify and quantify putative tomato allergens and correlate these reactions with previously conducted skin prick tests. Additionally, newly identified

putative tomato allergens could be identified and their importance as minor or major allergens were described and discussed.

Methods

Plant cultivation

The cultivation of plants for tomato production has been described in our two previous studies [11, 12]. Fruits were harvested at comparable red-ripe stage and stored at -80°C until their use in immunoblot analyses.

Tomato allergic subjects and clinical allergy tests

Participants were selected for our previous studies [11, 12] by the Allergy-Center-Charité Berlin, Germany, based on their positive history of adverse reactions to tomato (Table 1). The study was approved by the local ethic commission (EC-No. 1832/Si.258) and was conducted in accordance with the principals of the Declaration of Helsinki. All subjects gave written informed consent. Skin prick tests were carried out as described in the recommendations of GA²LEN [13] by prick-to-prick. Histamine dichloride (10 mg/mL; ALK-Abelló, Wedel, Germany) and saline solution (pH 7.4; ALK-Abelló) served as respective positive and negative controls. Wheal diameters were measured, and those ≥ 3 mm after 15 min were considered as positive in the absence of a reaction to the negative control. Tomato allergic subjects with a positive skin prick test reaction to at least one of the investigated tomato genotypes were further investigated. Skin prick tests were performed using fresh tomato fruit material of all described cultivars and genotypes ('Counter', '76R' and 'RMC'). The total and specific IgE to tomato were determined with the ImmunoCAP System (Thermo Fisher Scientific, Uppsala, Sweden) according to manufacturer's instructions. Serum samples of nine participants

Table 1. Characteristics of tomato allergic subjects (TAS). Table shows nine subjects with an anamnestic tomato allergy and positive skin prick test to tomato [11, 12]. Sera of these subjects were used for immunoblot analyses

Subject	Age (years)	Sex	Total IgE	Specific tomato IgE (kU/L)	Symptoms to tomato	Specific CCD IgE	Specific birch IgE (kU/L)	Specific grass IgE (kU/L)
TAS 1	40	M	114	2.02	GIT	nd	30.40	20.90
TAS 2	41	M	184	< 0.35	OAS	nd	29.30	43.60
TAS 3	36	M	197	0.86	OAS	nd	3.35	10.60
TAS 4	43	M	51	< 0.35	OAS	nd	24.30	4.00
TAS 5	38	M	155	< 0.35	R	nd	17.20	37.40
TAS 6	18	F	383	0.82	OAS	nd	37.10	69.70
TAS 7	18	F	234	nd	OAS	nd	nd	nd
TAS 8	36	F	405	0.92	GIT	no	> 100.00	2.71
TAS 9	36	F	255	1.17	OAS	nd	2.97	2.04

M, male; F, female; nd, not done; GIT, symptoms of the gastro-intestinal tract; OAS, oral allergy syndrome; R, rhinitis.

were stored at -20°C until their use in immunoblot analyses.

Protein extract preparation for immunoblot analyses

Tomato fruits were ground in a commercial blender and incubated in a buffer (1.4 M sucrose, 0.2 M KCl, 100 mM Tris pH 7.5, 50 mM ethylenediaminetetraacetic acid (EDTA), 2 mM phenylmethanesulfonyl fluoride (PMSF), 4% mercaptoethanol and 2% polyvinylpolypyrrolidone (PVPP)). After 30 min incubation on ice, samples were mixed with phenol, followed by a centrifugation step (10 000 g, 30 min, 4°C). Supernatants were precipitated with five volumes methanol, and pellets were solved in a buffer containing 7 M urea, 2 M thiourea, 2% carrier ampholytes (pH 2–4; Serva, Heidelberg, Germany) and 70 mM dithiothreitol (DTT). Protein concentrations were determined by Bradford assays (Proteome Factory AG, Berlin, Germany).

Two-dimensional gel electrophoresis and immunoblotting

Two-dimensional electrophoresis (2-DE) was carried out following the protocol by Klose and Kobalz [14]. Isoelectric focussing (IEF, first dimension) was conducted in vertical rod gels (9 M urea, 4% acrylamide, 0.3% piperazine diacrylamide, 5% glycerine, 2% carrier ampholyte (pH 2–11; Serva, Heidelberg, Germany), 0.06% N,N,N',N'-tetramethylethylenediamine (TEMED), 0.08% ammonium persulphate). 50 μg of protein was processed and focussed at 1,841 V. IEF gels were applied to SDS gels (15% acrylamide, 0.2% bisacrylamide, 375 mM Tris-HCl pH 8.8, 0.1% sodium dodecyl sulphate (SDS), 0.03% TEMED, 0.08% ammonium persulphate; second dimension) and covered with 0.5% agarose. Two replicate 2-DE separations were carried out for allergen localization. One gel was stained with FireSilver (Proteome Factory PS-2001, Berlin, Germany) for preparative applications. The other gel was used for Western blot analyses to detect the target proteins by immunostaining.

Blotting of 2-DE gels was performed with a Trans-Blot SD Semi-Dry Transfer Cell (Biorad, Munich, Germany) on an Immobilon-P membrane (PVDF, pore size 0.45 μm ; Millipore, Bedford, MA, USA) at a constant current of 5 V over night at 4°C using 25 mM Tris-HCl (pH 8.3) blotting buffer (192 mM glycine, 0.1% SDS, 20% methanol). For immunodetection of proteins, membranes were washed in TBST (20 mM Tris-HCl pH 7.5, 154 mM NaCl, 0.1% Tween-20) and blocked with TBST, containing 2% (w/v) bovine serum albumine (BSA) for 2 h. Membranes were incubated with subject's sera (diluted 1 : 10 in TBST, containing 1% (w/v) BSA) overnight and then incubated with

alkaline phosphatase-conjugated rabbit anti-human IgE (Sigma, Taufkirchen, Germany; diluted 1 : 1500 in TBST, containing 1% (w/v) BSA) at room temperature for 2 h. Finally, retained antibodies were detected by incubating with Fast Red/Naphtol (Sigma) for 30 min. Membranes were washed in TBST between all incubation steps.

Immunoblots were carried out with nine tomato allergic subjects and one non-allergic subject, as a negative control.

Identification of putative tomato allergens

Protein identification was performed by nanoLC-ESI-MS/MS. The MS system consists of an Agilent 1100 nanoLC system (Agilent, Böblingen, Germany), PicoTip emitter (New Objective, Woburn, USA) and an Esquire 3000 plus ion trap MS (Bruker, Bremen, Germany). Protein spots were in-gel digested by trypsin (Promega, Mannheim, Germany) and applied to nanoLC-ESI-MS/MS. Peptides were trapped and desalted on an enrichment column (Zorbax SB C18, $0.3 \times 5 \text{ mm}$, Agilent, Böblingen, Germany) using 1% acetonitrile/0.1% formic acid solution for 5 min and subsequently separated on a Zorbax 300 SB C18, $75 \mu\text{m} \times 150 \text{ mm}$ column (Agilent, Böblingen, Germany) using an acetonitrile/0.1% formic acid gradient from 5% to 40% acetonitrile within 40 min. MS spectra were automatically taken by Esquire 3000 plus according to manufacturer's instrument settings for nanoLC-ESI-MS/MS analyses. Proteins were identified using MS/MS ion search of Mascot search engine (Matrix Science, London, UK) and a protein database (National Center for Biotechnology Information (NCBI), Bethesda, MD, USA).

Ion charge in search parameters for ions from ESI-MS/MS data acquisition was set to '1+, 2+ or 3+' according to the instrument's and method's common charge state distribution. Mascot expresses the probability that peptides match at random to a given protein by a probability score. A score larger than 57 indicates identity or extensive homology ($P = 0.05$).

Spot (putative tomato allergens) quantity and percentage calculation

Identified putative allergens, positively reacting with the subject's sera, were counted on immunoblots for each single subject with each tomato genotype and growth condition. These positive spots were summed up for each immunoblot and are given in spot quantity. Additionally, for each identified putative allergen, positive spots of all immunoblots were counted and the percentage of positive reacting subjects was calculated.

Correlation analysis

Correlation analysis of skin prick test results (wheal diameter) vs. spot quantity of immunoblots was carried out using the program Statistica (version 9, Tulsa, OK, USA).

Results

Identification of tomato allergens

Sera of nine tomato allergic subjects were used to identify putative tomato allergens. A non-allergic subject serum served as a negative control and showed no

positive spots on the positions of the identified proteins (data not shown).

To identify proteins, putatively responsible for the allergic reaction of tomato allergic subjects, proteins representing the positive spots on the immunoblots were analysed by mass spectrometry. We could identify ten putative tomato allergens; among those, five which are newly described here (Fig. 1 and Table 2): an endo- β -mannanase (MAN), a pectinesterase inhibitor U1 (PEIU1), two proteins similar to a pectinacetyltransferase (PAE) and an aspartyl protease family protein (AP), and a protein of unknown function (unknown). On the contrary, the pectinmethylesterases PME1.9 and PME2.1 [15, 16], the polygalacturonase PG [15, 16], the

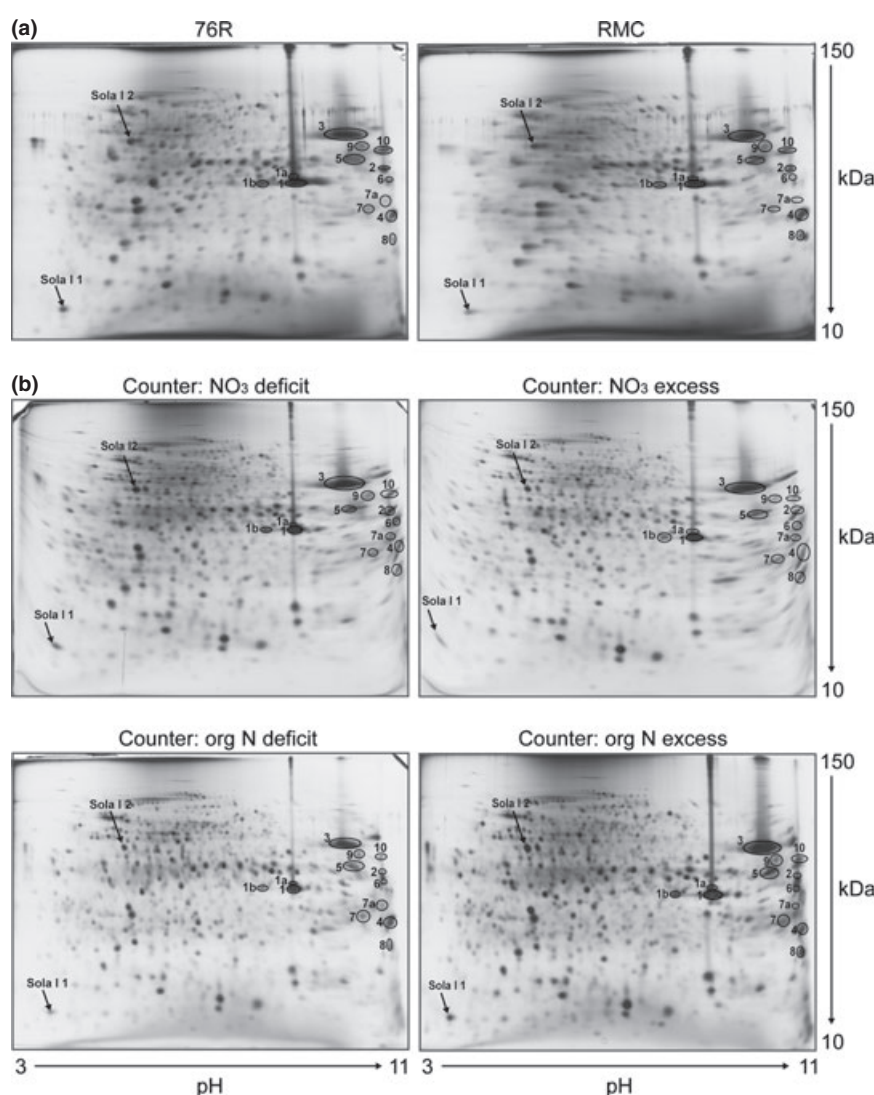


Fig. 1. 2-D protein gels with identified putative tomato allergens (circled) and the confirmed tomato allergens, Sola 1.1 and Sola 1.2 (arrows). Proteins were separated due to their molecular weights (kDa) and isoelectric points (pH). Proteins detected in immunoblots with the sera of nine tomato allergic subjects and identified by mass spectrometry are marked with numbers and listed in Table 2. (a) Proteins were extracted from fruits of the tomato genotype 76R and its isogenic mycorrhizal mutant RMC. (b) Proteins were extracted from fruits of the tomato cultivar Counter cultivated with different nitrogen fertilizers (NO_3 and organic nitrogen in deficit or excess, respectively).

Table 2. Putative allergens identified on immunoblots with tomato fruits of the two tomato genotypes 76R and RMC and the cultivar Counter treated with different nitrogen fertilizers. Protein extracts were separated by 2-D gel electrophoresis and analysed by immunoblotting with sera of nine tomato allergic subjects. Protein spots which showed a reaction were eluted from parallel gels and identified using mass spectrometry. Proteins are indicated by numbers and shown in Fig. 1

Spot no. in gel	Name	Accession number	Molecular weight/ Isoelectric point (theoretical)	Molecular weight/ Isoelectric point (in gel)	Score/ Sequence identity (%)	Known as allergen in tomato	Abbreviation
1 (1a,1b)	Pectinmethyl-esterase 1	gi 6174913	60/6.3	27/8.1	470/20	yes	PME1.9
2	Pectinmethyl-esterase 2.1	gi 2507165	60.5/8.7	30/9.8	323/15	yes	PME2.1
3	Polygalacturonase 2	gi 129939	50/6.4	37/9	758/30	yes	PG
4	NP24	gi 170467	25.7/8.3	19/9.8	111/8	yes	NP24
5	Endo- β -mannanase	gi 66360069	42.4/8.8	33/9	549/35	no	MAN
6	Pectinesterase inhibitor U1	gi 6093740	64.1/9	29/10	330/16	no	PEIU1
7 (7a)	Unknown	gi 924626	25.2/9.4	24/9.8	304/28	no	—
8	Similar to 11S globulin isoform 4 (<i>Sesamum indicum</i>)	EST: BP891368 (TC241385)	18.3/10.1	18/9.8	282/43	yes	11SG
9	Similar to aspartyl protease family protein (<i>Arabidopsis thaliana</i>)	EST: A1484598 (gi 15232503)	16.1/10.2	34/9.4	303/38	no	AP
10	Similar to pectinacetylsterase precursor (<i>Vigna radiata</i>)	EST: BP891840 (gi 1431629)	17.8/8.2	34/10	156/40	no	PAE

thaumatin-like protein NP24 [15] and a protein similar to the 11S globulin (11SG) [17] have already been described as putative tomato allergens. Even when theoretical molecular weights of the identified proteins were often not in concurrence with the values experimentally achieved, identifications are reliable due to high sequence coverage and scores with the database entries obtained in Mascot or Blast searches (Table 2). Differences in molecular weights can be due to, for example, partly processed proteins. The two confirmed tomato allergens Sola 1 1 and Sola 1 2 could not be detected as positive reactions with any of the subject's sera. However, they were present in the extracts of all genotypes and growth conditions (Fig. 1, arrows), and we observed reactions to these well-known tomato allergens in other studies [7, 18].

Distribution of minor and major putative tomato allergens

Figure 2 shows the percentage of tomato allergic subjects' IgE, reacting with the respective putative tomato allergens. The IgEs of all subjects interacted with eight of ten proteins, but reactions to the proteins were dependent on genotype and growth condition. Only PEIU1 and the protein of unknown function seemed not to interact with every serum. Therefore, a statement about minor and major allergens is here difficult to render. However, for the current cohort of subjects and

for the genotypes and growth conditions used for fruit production, the known tomato allergens pectinesterase (PME1.9) and the thaumatin-like protein (NP24) were the most prominent ones.

Correlation analysis of skin prick test results vs. spot quantity of immunoblots

To compare the highly variable wheal diameters in skin prick tests of the tomato allergic subjects with the number of positive reacting spots in the corresponding immunoblots, a correlation analysis was performed. In total, 54 immunoblots were compared with 54 skin prick test results (nine subjects $\times$ six treatments). No significant correlations were observed neither within a particular tomato genotype or growth condition (data not shown) nor over all genotypes and growth conditions ($r = 0.036$, Fig. 3).

Discussion

The basis for the current study was the observation that tomato allergic subjects showed highly variable reactions in clinical allergy tests even to tomato fruits from the same genotype, grown under identical conditions. This observation led to the hypothesis that the IgEs of different subjects interact with different tomato allergens and that this, in turn, results in more or less serious allergic reactions. Therefore, previously achieved

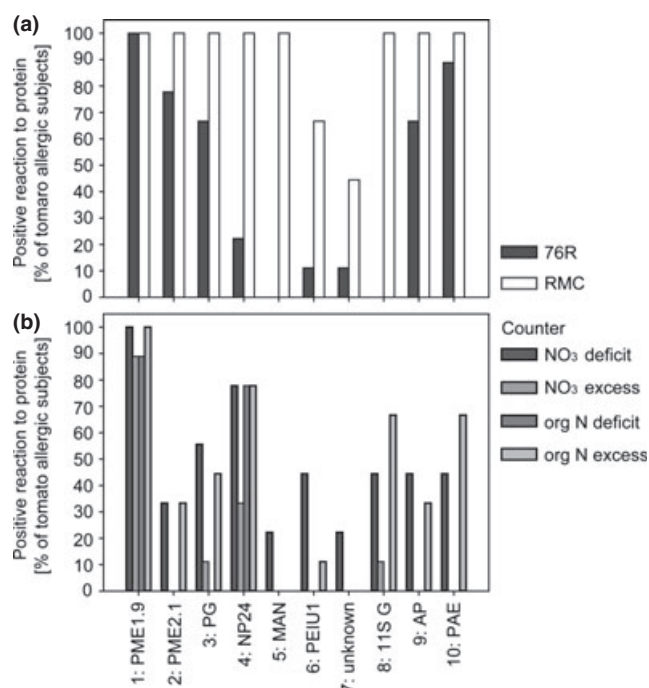


Fig. 2. Positive interactions of IgEs to putative tomato allergens on immunoblots. Values are given in percent of nine tomato allergic subjects (TAS). (a) Positive interactions to proteins of the two tomato genotypes 76R and its isogenic mycorrhizal mutant RMC. (b) Positive interactions to proteins of the tomato cultivar Counter fertilized with NO_3 or organic nitrogen (org N) in deficit or excess. Abbreviations: PME1.9: pectinesterase 1.9, PME2.1: pectinesterase 2.1, PG: polygalacturonase 2, NP24: thaumatin-like protein, MAN: endo- β -mannanase, PEI U1: pectinesterase inhibitor U1, unknown: protein of unknown function, 11SG: similar to 11S globulin, AP: similar to aspartyl protease family protein, PAE: similar to pectinacetylase.

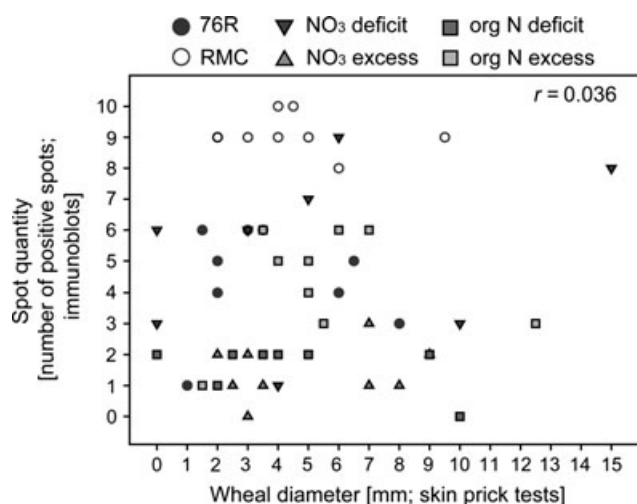


Fig. 3. Comparison of skin prick test results and spot quantity on immunoblots. Correlation analyses were carried out over nine tomato allergic subjects with the two tomato genotypes 76R and RMC and with fruits from tomato cultivar Counter cultivated with different N fertilizers. Wheal diameters [mm] of skin prick tests were correlated with the number of positive spots of the corresponding immunoblots.

skin prick test data [11, 12] were correlated with the amount of positive protein-IgE interactions on immunoblots. Although the IgEs of the subjects indeed reacted with different allergens among the tomato fruit extracts, no correlation to the reactions in skin prick tests could be detected. This indicates that variations in allergic reactions among individual subjects cannot simply be explained by the amount of proteins they react to.

Thus, positive protein spots on the immunoblots were additionally qualified, and this resulted in the identification of ten putative tomato allergens. Five of them have been previously described (www.allergome.org), and five were detected in the current study for the first time.

Different pectinesterases reacted with subjects' IgEs. Two of them, PME1.9 and PME2.1, have been described as putative tomato allergens before [15, 16]. Furthermore, a pectinesterase inhibitor U1 and a pectinacetylase were identified here as new putative tomato allergens. Pectinesterases have been first identified as allergens in tomato fruits, but in the meantime, they were also detected among allergens from ash tree and grass [19, 20]. Therefore, the IgE interaction pattern of these proteins could very well be based on the cross-reactivity between tomato and other plant species [21]. The polygalacturonase is an important tomato allergen [22] and has been always identified as one of the major allergens in our previous analyses. Pectinesterases and polygalacturonases are known to have N-glycosylation sites, also called cross-reactive carbohydrate determinants (CCD) [7, 23]. Some allergic subjects are known to possess specific IgE antibodies against these CCDs although its clinical relevance is still discussed [23–25]. As some of the newly identified putative tomato allergens might also be glycoproteins, harbouring CCDs, the presence of anti-CCD specific antibodies was examined in one subject's serum (TAS 8). As well as in our previous study [7], we could not observe any difference in IgE-binding pattern which might be based on CCDs. TAS 8 had no specific CCD IgEs and showed reactions to all identified putative tomato allergens, except for the protein of unknown function. This denotes the uncertainty of being a clinically relevant allergen, is only true for one protein. All other newly identified allergen candidates seem not to be based on CCD-IgE cross-linkages.

The detected endo- β -mannanase has not yet been described to be an allergen of plants. However, allergenic extracts from mites contain a number of enzymes, including a mannosidase [26] and *Aspergillus fumigatus* also harbours an allergen which was identified as this enzyme [27]. Mannosidases as well as pectinesterases and polygalacturonases are functionally involved in the fruit ripening process [28–30]. Besides those, an aspartyl protease family protein (also known as nucleoid

DNA-binding-like protein) has never been described as a putative allergen. The function of this protein in tomato is unknown, but the homologue in tobacco possesses proteolytic activity and might be involved in the degradation of denatured proteins under stress conditions [31]. Another stress- or defence-related protein, which interacted with the subjects' IgEs, was the thaumatin-like protein NP24. It belongs to the pathogenesis-related protein family PR-5 and is a known allergen in many fruits and vegetables [32]. In tobacco, this protein inhibits hyphal growth and spore germination of a large number of economically important plant pathogens [33]. The seed storage protein 11S globulin was recently described as a putative tomato allergen [17], and a similar protein could be again identified with the IgEs of these subjects.

Although many known and new putative allergens were detected in the present study, the four confirmed tomato allergens (by the International Union of Immunological Societies; www.allergen.org), Sola 1 1 (profilin), Sola 1 2 (β -fructofuranosidase), Sola 1 3 (lipid transfer protein) and Sola 1 4 (Bet v 1 homologue), seemed not to interact in these immunoblots. Tomato allergic subjects, originating from Northern Europe, are often sensitized through birch or grass pollen, and allergic symptoms after tomato ingestion are mainly caused by cross-reactive allergens. Those allergic reactions are dominated by oral allergy symptoms and basically occur after the ingestion of raw material, indicating sensitizations to mainly unstable allergens [34–36]. Considering that this particularly applies to the investigated subjects, here newly identified putative allergens can probably not resist heat or proteolysis [37]. For this reason, these subjects might not be sensitized to Sola 1 3, a heat and proteolysis stable lipid transfer protein, whose sensitization is mainly independent from pollen cross-reactivity and often cause severe systemic reactions [35, 38]. In addition, lipid transfer protein sensitization is known to be more common in the Mediterranean area [35] and less among subjects, coming from Berlin and surroundings. Surprisingly, immunoblots showed also no interaction of subjects' sera with Sola 1 1 and Sola 1 4, even if there is very low prevalence of tomato allergy in the absence of birch pollen or profilin sensitization [38, 39]. However, Sola 1 1 was a dominant allergen in our previous studies [7, 18] and definitely is present in the protein extracts (Fig. 1). Hence, investigated subjects' IgEs probably interacted with Sola 1 1, and the absent detection on immunoblots was caused by the less sensitive alkaline phosphatase-conjugated secondary antibody. In contrast, Sola 1 4 has never been detected in one of our previous immunoblot analyses; therefore, it remains unknown if this absent detection resulted from absent sensitizations, if it was a problem

of detection or if this protein was less abundant in the investigated tomato fruit protein extracts. Sola 1 2 is commonly detected as a major tomato allergen [7, 18], and invisible IgE interactions with any of the single subject's sera were probably based on the above-mentioned detection difficulties with the secondary antibody. However, Sola 1 2 is discussed to be a 'true' food allergen because of its glycan structures important for its allergenic activity [40, 41]. Absent interactions with Sola 1 1 and Sola 1 2, based on possible technical problems, can therefore not be completely excluded as one reason for the low correlation rate between immunoblot analyses and skin prick test results, even if Sola 1 1 and Sola 1 2 sensitization can be expected for all of the investigated subjects.

Additionally, single subjects showed interactions with certain proteins in immunoblots but no reaction at all to the same tomato fruits in skin prick tests and vice versa. On the one hand, this might be explained by false-positive reactions in immunoblots. Positive IgE-allergen cross-linkages on immunoblots can be based on only one allergen epitope. In contrast, at least two epitopes are necessary for the cross-linking to specific IgE receptors to provoke positive reactions in skin prick tests [42, 43]. Furthermore, specific IgEs to a certain protein, detected in immunoblots, do not have to be directly associated with the biological activity of the protein. The affinity between IgEs and allergens is the basis for their biological activity and their capability to cause symptoms of allergy [44]. Positive reactions in immunoblots probably imply lower affinity rates than required to trigger allergic symptoms. Therefore, only proteins with a positive interaction on the immunoblot in combination with a positive skin prick test for the respective tomato fruit were identified as new putative tomato allergens. On the contrary, the absence of any IgE-protein interaction on the immunoblot in combination with a positive skin prick test reaction might be explained by low-abundant allergens or allergen epitopes, which are denatured or degraded, in the protein extracts used for the immunoblots.

Interestingly, some commercial ImmunoCAP specific tomato IgE measurements of the tomato allergic subjects showed negative results. Possibly, these commercial extracts contain, if at all, only minor amounts of respective allergens. It is known that these commercial extracts sometimes have a relatively low sensitivity probably due to denatured or degraded proteins, occurring after certain protein extraction methods [45]. This might explain positive skin prick tests and immunoblots to certain putative allergens and, at the same time, negative specific IgE to tomato in ImmunoCAP tests.

Within the cohort of this study, IgEs of every investigated serum showed a reaction with eight of ten of identified putative tomato allergens. Therefore, we could

not determine candidate proteins, being responsible for the high individuality in clinical reactions. Interestingly, nearly every identified putative allergen of the mycorrhizal mutant RMC provoked reactions with 100% of the subjects' sera. In contrast, the pectinmethylesterase 1.9 was the only protein of the corresponding wild-type 76R which interacted with 100% of the subjects' sera (Fig. 2). This might be explained by different expression pattern, however, not obviously visible neither in RNA accumulation of respective genes [11] nor on the protein gels (Fig. 1). Another possibility might be genotype-dependent modifications of the proteins. Anyway, it only resulted in slightly higher skin prick test results to the mutant RMC which were in summary over all subjects not significant [11]. Pectinmethylesterase 1.9 and the thaumatin-like protein NP24 represent the most prominent putative allergens in this study. Pectinmethylesterase 1.9 from tomatoes of all genotypes and growth conditions interacted with at least 88% of the subjects' sera. Reactions to NP24 were indeed less frequent, but except for the immunoblots with the genotype 76R and the cultivar Counter fertilized with an excess of NO₃, more than 80% of the subjects' sera showed a reaction to this protein.

Taken together, this study showed that high inter-individual differences in allergic reactions of tomato allergic subjects cannot simply be explained by the quantity or quality of putative allergens their sera interact with. Subjects showed individual IgE-binding profiles, and some proteins could represent major allergens, but this seemed to be dependent on the tomato genotype and respective growth conditions. The five new tomato allergen candidates detected in this study for the first time will be investigated in further studies, including their expression as recombinant proteins, to confirm their allergenicity.

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Conflict of interest

All authors declare that there is no conflict of interest regarding the investigation described in the manuscript.

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