

RESEARCH ARTICLE

Identification of Sola l 4 as Bet v 1 homologous pathogenesis related-10 allergen in tomato fruits

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Scope: The aim was to investigate the potential contribution of a major birch pollen Bet v 1-homologous allergen to birch pollen-associated tomato fruit allergy.

Methods and results: Two isoforms of a Bet v 1-homologous protein (designated Sola l 4) from tomato fruit were identified by cDNA-cloning and produced as recombinant proteins. Allergen-specific IgE levels to tomato, birch pollen, Bet v 1, and Sola l 4 were determined in birch pollen allergic patients with allergy or tolerance to tomato. Sola l 4 was recognized in 76% of birch/tomato allergic patients, while tomato- and Bet v 1-specific IgE was detectable in 64% and 81% of sera. Almost all patients sensitized to Bet v 1 reacted with Sola l 4. Both Sola l 4 isoforms displayed allergenic potency and IgE-cross-reactivity with Bet v 1 as investigated by competitive ELISA and in vitro mediator release assay. Nevertheless, the reactivity pattern of patients' sera was diverse.

Conclusion: Sola l 4, a novel pathogenesis related-10 protein, qualifies as major allergen in tomato fruits. Data suggest Sola l 4 as class II allergen. IgE-testing using Sola l 4 showed low clinical specificity, but high sensitivity in tomato allergic patients and will further improve component-resolved allergy diagnosis.

Keywords:

Bet v 1 / Birch pollen allergy / Pathogenesis related-10 protein / Sola l 4 / Tomato allergy

Received: August 23, 2013

Revised: November 22, 2013

Accepted: December 17, 2013

1 Introduction

The consumption of tomatoes (*Solanum lycopersicum*) has doubled over the last decades and has been reported to cause adverse IgE-mediated allergic reactions [1]. Studies on the prevalence of tomato allergy using oral challenge tests, rather than reports on sensitization, are limited. In line with this, patients reporting tomato allergy frequently did not show tomato-specific IgE, likely due to non-IgE mediated adverse reactions. Nevertheless, 3.3% of food allergic subjects worldwide [2], and 16–33% of Italian patients with fruit and/or veg-

etable allergy show IgE-binding to tomatoes [3–5]. Patients with reactivity to tomatoes are frequently sensitized to aeroallergens suggesting cross-reactivity with pollen allergens [6] due to putative homologous cross-reactive proteins. In a study published by Foetisch et al. [7], 9% of birch pollen allergic patients reported adverse reactions to tomato, and de Martino et al. [8] showed that 39% of grass pollen allergic children are sensitized to tomato. Furthermore, cross-reactivity to tomato was described for up to 28% of latex allergic patients [9]. Manifestation of tomato allergy mostly includes mild symptoms, such as oral allergy syndrome (OAS), as well as rhinitis and abdominal pain [6, 7], but also urticaria and contact dermatitis, anaphylactic reactions, and exercise-induced symptoms are described [10–12].

Despite the sensitization pattern, showing several tomato allergens likely to be involved in tomato allergy [7, 13], only three tomato allergens have so far been officially accepted by the IUIS Allergen Nomenclature Subcommittee. At least 32% of food allergic [14] and approximately 22% of tomato

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Abbreviations: aa-id, amino acid identity; CD, circular dichroism; OAS, oral allergy syndrome; PR, pathogenesis related

allergic patients [15] are sensitized to tomato profilin Sola l 1. 17% of tomato allergic patients are sensitized to invertase (β -fructofuranosidase) Sola l 2 [16, 17] and up to 15% of Italian tomato allergic patients were mono-sensitized to the non-specific lipid transfer protein from tomato, Sola l 3 [1, 18]. In a multicenter study, about 35% of pollen-fruit allergic patients had IgE to Sola l 3 using a microarray assay [19]. Moreover, pectinesterase, polygalacturonase 2A, superoxide-dismutase [7, 13], an osmotin-like protein, glucanase, and chitinase, as well as a 9 kDa heat-labile and pepsin-resistant protein are discussed as further tomato allergens [20–23].

Pollen associated food allergy, including tomato allergy, is considered to be elicited by cross-reactive allergenic structures, e.g. profilins, cross-reactive carbohydrates and in particular by Bet v 1-like proteins of the pathogenesis-related protein 10 (PR-10) family. In Central Europe, Bet v 1, the major allergen in birch pollen, has been described as the primary sensitizer for cross-reactivity to PR-10 proteins from fruits and vegetables [24]. Although multiple sensitization profiles of tomato allergic patients have been shown using tomato extract [17], no Bet v 1 homologous PR-10 protein from tomato with allergenic properties is described so far, even though there is some evidence for the presence of this gene family in tomatoes [22, 23]. The objective of the present study was the identification of Bet v 1-homologous PR-10 proteins in tomato fruits and the investigation of their allergenic properties.

2 Material and methods

2.1 Patients' sera and ImmunoCAP™ testing

Overall 68 patients, 42 birch pollen allergic patients with allergy to fresh tomato fruits (group 1) and 26 birch pollen allergic patients with tolerance to tomato (group 2) were included in the study (Table 1). Birch pollen allergic patients were selected by clinical symptoms, e.g. seasonal rhinoconjunctivitis allergica and asthmatic symptoms in response to early flowering trees (including birch, alder, and hazel) and sensitization to birch pollen, determined by positive skin test and/or ImmunoCAP™ test with birch pollen specific IgE >0.35 kU_A/L. Tomato allergic patients were selected by clear clinical history upon ingestion of raw tomatoes. OAS was the most frequently (73.8%, $n = 31$) observed symptom. Systemic symptoms, predominantly skin reactions were reported in 9 patients (21%). Approval by the local ethics committee (Faculty of Medicine, University of Erlangen-Nuremberg, No. 3344) for this study and written informed consent of all participants were obtained. Prick-to-prick tests in tomato allergic patients were performed as described [25]. 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 minutes and sizes of 3 mm or greater were considered positive.

2.2 cDNA cloning, expression, and purification of Sola l 4 isoforms

RNA from the commercially available tomato variety *Verona* was prepared by using the TRIzol® Reagent (Fisher Scientific, Schwerte, Germany), subsequently the First-Strand cDNA Synthesis Kit (GE Healthcare, Freiburg, Germany) was applied according to the manufacturer's instructions. For PCR-based amplification, primer Sola l 4.01(+) 5'-ATG GGT GTG AAT ACC TAT ACT T-3', Sola l 4.02(+) 5'-ATG GGT GTA AAC ACC TTT ACT-3' (forward), and Sola l 4.01(-) 5'-TCA AGC GTA GAC AGA AGG ATT C-3', Sola l 4.02(-) 5'-TTA AGC GTA GAC AGA AGG ATT AG-3' (reverse) were designed using two Bet v 1-homologous sequences (GenBank Acc. Nos. AK247106 and AK224718) included in the tomato gene database MiBASE. PCR products were cloned into pCRII-Topo vector (Fisher Scientific) for sequence verification, done by Eurofins (Ebersberg, Germany) using the cycle sequencing technology (dideoxy chain termination/cycle sequencing) on an ABI 3730XL sequencer. Full-length cDNA was obtained by 5' and 3'-rapid amplification of cDNA ends (Fisher Scientific) according to manufacturer's instructions. Both Sola l 4 cDNA's were subcloned via NdeI restriction sites into the expression vector pET15b (Merck, Darmstadt, Germany), encoding a N-terminal hexa-histidine (His)₆-tag using the In-Fusion HD cloning system (Clontech, Saint-Germain-en-Laye, France). The Sola l 4-pET15b constructs were transformed into BL21(DE3) competent *E. coli* cells (Merck). Protein synthesis was induced for 14 h at 23°C by adding 1 mM isopropyl- β -D-thio-galactopyranoside at an optical density of OD_{600 nm} = 0.6–0.8. Purification of (His)₆-tagged Sola l 4 was performed by immobilized metal affinity chromatography with Ni-NTA under native conditions using a His GraviTrap column (GE Healthcare) according to the manufacturer's instructions. Recombinant (r) Bet v 1.0101 (Acc. No. X15877) was prepared as described elsewhere [26].

2.3 Physicochemical analysis

Circular dichroism (CD) spectroscopy of the recombinant proteins was performed on a Jasco J-810 spectropolarimeter (Jasco, Groß-Umstadt, Germany) equipped with a CDF-426S Peltier temperature control system, interfaced with a Julabo F200 water bath (Julabo Labortechnik GmbH, Seelbach, Germany). CD spectra in the range of 185–255 nm were recorded at 20°C using a scanning speed of 50 nm/min, a step width of 0.2 nm and a bandwidth of 1 nm. To increase the S/N, ten scans were accumulated. Measurements were performed in a 1 mm path length quartz cell cuvette (200 μ L, Hellma, Müllheim, Germany) at protein molarities of about 5 μ M in 10 mM potassium phosphate buffer (pH 7.0). The mean residue ellipticity ([θ]_{m.r.w.}) was calculated from the measured ellipticity [θ] according to [θ]_{m.r.w.} = $10 \times \theta / \text{cdN}$, where θ denotes the measured ellipticity in millidegrees,

Table 1. Birch pollen allergic patients with clinical history of allergy (nos. 1–42) or tolerance (nos. 43–68) to tomato. Reported clinical symptoms upon ingestion of tomato, specific IgE values to tomato and birch extract, rBet v 1.01 and rSola I 4.02 are indicated. Sera used for IgE-inhibition and histamine release assays shown in the results part are marked in bold. OAS: oral allergy syndrome; RCA: rhinoconjunctivitis allergica

Patient no	Clinical symptoms		Skin prick test MWD		CAP [kU _A /L] (CAP class)			
	Tomato	Birch	Tomato	Birch	Tomato (t25)	Birch (t3)	Be t v 1.01 (t215)	rSola I 4.02
1	OAS	Seasonal RCA	6 mm	12 mm	3.08 (2)	100 (6)	159.4 (6)	25.9 (4)
2	Contact urticaria stage I	Seasonal RCA with asthmatic symptoms	7 mm	7 mm	2.70 (2)	25.1 (4)	23.7 (4)	4.90 (3)
3	Facial erythema	Seasonal RCA	nd	nd	0.58 (1)	63.9 (5)	75.4 (5)	20.9 (4)
4	OAS	Seasonal RCA	nd	nd	0.67 (1)	38.8 (4)	31.3 (4)	7.70 (3)
5	OAS	Seasonal RCA	7 mm	7 mm	11.9 (3)	>100 (6)	>100 (6)	>100 (6)
6	OAS, dyspnoea	Contact urticaria	2 mm	7 mm	0.57 (1)	100 (6)	100 (6)	100 (6)
7	OAS	Seasonal RCA with asthmatic symptoms	3 mm	7 mm	1.78 (2)	1.0 (2)	0.02 (0)	0.19 (0)
8	Contact urticaria stage I	Seasonal RCA	7 mm	7 mm	0.42 (1)	35.9 (4)	28.9 (4)	11.3 (3)
9	OAS	Seasonal RCA with asthmatic symptoms	nd	nd	0.54 (1)	50.4 (5)	61.6 (5)	0.57 (1)
10	OAS	Seasonal RCA	6 mm	3 mm	3.90 (3)	29.7 (4)	22.41 (4)	5.91 (3)
11	OAS	Seasonal RCA	3 mm	7 mm	0.14 (0)	0.31 (0)	0 (0)	0.20 (0)
12	OAS	Seasonal RCA	4 mm	3 mm	0.07 (0)	0.1 (0)	0.01 (0)	0.16 (0)
13	OAS	Seasonal RCA	4 mm	3 mm	0.05 (0)	0.04 (0)	0.02 (0)	0.19 (0)
14	Gastrointestinal symptoms	Discretely seasonal RCA	4 mm	4 mm	0.05 (0)	23.2 (4)	23.5 (4)	2.14 (2)
15	OAS	Seasonal RCA with asthmatic symptoms	4 mm	7 mm	1.63 (2)	37.9 (4)	34.6 (4)	13.0 (3)
16	OAS	Seasonal RCA with asthmatic symptoms	7 mm	7 mm	2.29 (2)	14.2 (3)	16.6 (3)	1.3 (2)
17	OAS	Seasonal RCA	7 mm	4 mm	0.37 (1)	0.55 (1)	0.02 (0)	1.45 (2)
18	Urticaria	Seasonal RCA	4 mm	6 mm	1.21 (2)	8.73 (3)	4.9 (3)	2.52 (2)
19	OAS	Seasonal RCA	3 mm	7 mm	0.06 (0)	8.58 (3)	7.50 (3)	3.02 (2)
20	OAS	Seasonal RCA	Negative	4 mm	0.04 (0)	7.80 (3)	9.16 (3)	2.24 (2)
21	OAS	Seasonal RCA	2 mm	4 mm	1.48 (2)	28.5 (4)	27.0 (4)	1.80 (2)
22	Urticaria stage I, gastrointestinal symptoms	Seasonal RCA	4 mm	3 mm	1.20 (2)	0.31 (0)	0.02 (0)	0.21 (0)
23	OAS	Seasonal RCA	2 mm (late phase reaction)	7 mm	0.16 (0)	27.2 (4)	29.0 (4)	1.71 (2)
24	OAS	Seasonal RCA with asthmatic symp to ms	3 mm	7 mm	0.50 (1)	0.71 (2)	0.01 (0)	0.16 (0)
25	OAS	RCA in May since 2 years	Negative	7 mm	0.74 (2)	2.56 (2)	2.18 (2)	1.50 (2)
26	Contact urticaria stage I, OAS	Seasonal RCA	3 mm	7 mm	9.49 (3)	100 (6)	100 (6)	55.7 (5)
27	OAS	Seasonal RCA	nd	>3 mm	2.08 (2)	35.9 (4)	43.4 (4)	3.71 (3)
28	Facial urticaria, rhinitis, eczema	Asthma bronchiale	nd	nd	0.94 (2)	>100 (6)	>100 (6)	13.1 (3)
29	Sickness, stomach pain	Seasonal rhinitis	>3 mm	nd	0.03 (0)	0.71 (2)	0.63 (1)	0.20 (0)

Table 1. Continued

Patient no	Clinical symptoms	Skin prick test MWD		CAP [kU _A /L] (CAP class)			
		Tomato	Birch	Tomato (t25)	Birch (t3)	Be t v 1.01 (t215)	rSola l 4.02
30	OAS	>3 mm	nd	1.35 (2)	0.95 (2)	0.76 (2)	0.19 (0)
31	OAS	>3 mm	nd	57.8 (5)	> 100 (6)	> 100 (6)	> 100 (6)
32	OAS	nd	nd	1.27 (2)	54.3 (5)	71.8 (5)	30.1 (4)
33	OAS	nd	nd	15.5 (3)	43.5 (4)	31.2 (4)	0.23 (0)
34	Urticaria	Negative	nd	<0.35 (0)	1.1 (2)	0.04 (0)	0.17 (0)
35	OAS, joint swelling	nd	nd	<0.35 (0)	6.1 (3)	7.69 (3)	1.06 (2)
36	Stomach ache, diarrhea, atopic dermatitis, OAS	nd	nd	0.05 (0)	20.9 (4)	26.5 (4)	6.12 (3)
37	Skin itching	nd	nd	0.16 (0)	17 (3)	18 (4)	1.21 (2)
38	OAS	nd	nd	0.05 (0)	7.35 (3)	9.92 (3)	0.59 (1)
39	Lip swelling	nd	nd	0.06 (0)	15.8 (3)	16.1 (3)	2.76 (2)
40	OAS	nd	nd	1.45 (2)	19.4 (4)	20.1 (4)	7.97 (3)
41	OAS	nd	nd	4.31 (3)	24.5 (4)	15.8 (3)	10.9 (3)
42	OAS	nd	3 mm	0.08 (0)	15.0 (3)	14.0 (3)	4.08 (3)
43	None	nd	++	0.44 (1)	42 (4)	58.5 (5)	12.1 (3)
44	None	nd	+	1.58 (2)	>100 (6)	>100 (6)	61.1 (5)
45	None	nd	+	0.03 (0)	30 (4)	27.9 (4)	8.54 (3)
46	None	nd	+	0.06 (0)	52 (5)	35.4 (4)	2.01 (2)
47	None	nd	nd	2.28 (2)	65.4 (5)	48.3 (4)	10.1 (3)
48	None	nd	+	2.27 (2)	49.7 (4)	29.5 (4)	24.3 (4)
49	None	nd	+	0.27 (0)	60.9 (5)	77.7 (5)	14.3 (3)
50	None	nd	nd	0.77 (2)	47.7 (4)	25.4 (4)	14.9 (3)
51	None	Negative	++	0 (0)	19.4 (4)	20.6 (4)	2.85 (2)
52	None	nd	nd	16.9 (3)	41.7 (4)	37.1 (4)	18.1 (4)
53	None	nd	nd	0.25 (0)	35.1 (4)	44.2 (4)	11.6 (3)
54	None	Negative	+++	0.07 (0)	20.3 (4)	23.3 (4)	7.57 (3)
55	None	nd	+++	0.07 (0)	98.2 (5)	75.9 (5)	25.8 (4)
56	None	nd	nd	0.79 (2)	>100 (6)	>100 (6)	56.6 (5)
57	None	nd	++	0.09 (0)	32.6 (4)	26.2 (4)	10.1 (3)
58	None	nd	nd	0.03 (0)	24.1 (4)	26 (4)	10.6 (3)
59	None	nd	nd	0.52 (1)	100 (6)	94.2 (5)	20.8 (4)
60	None	nd	nd	0.37 (1)	78.1 (5)	61.7 (5)	50.2 (5)
61	None	nd	nd	1.65 (2)	71.2 (5)	84.6 (5)	5.55 (3)
62	None	++	+++	1.13 (2)	82.8 (5)	70.6 (5)	49.3 (4)
63	None	+	+++	1.1 (2)	80.0 (5)	79.2 (5)	31.6 (4)
64	None	nd	nd	0.02 (0)	20.1 (4)	20.6 (4)	2.47 (2)
65	None	nd	nd	0.03 (0)	57.9 (5)	100 (6)	19.3 (4)
66	None	nd	nd	0.04 (0)	32.1 (4)	34.1 (4)	7.11 (3)
67	None	nd	nd	0.09 (0)	32.5 (4)	23.8 (4)	5.12 (3)
68	None	nd	nd	0.04 (0)	27 (4)	25.6 (4)	11.2 (3)

MWD, mean wheal diameter.

c the protein concentration in millimolar, d the light path in centimeter and N is the number of amino acid residues.

To confirm the identity of rSola l 4.0101 and 4.0201, LC-MS was performed. Recombinant proteins were excised from Coomassie stained SDS-PAGE gels and further processed as described elsewhere [27] with slight modifications. Peptides were eluted with 25 mM NH_4HCO_3 ; 10% ACN and the digestions were stopped by adding 5% formic acid. The peptides were analyzed by using a nano-ultra performance LC system coupled to a nano-ESI-MS (nano Acquity UPLC nanoESI Synapt-MS, Waters, Milford, USA) with a 5 μm symmetry 180 $\mu\text{m} \times 20 \text{ mm}$ c18 precolumn and a 1.7 μm BEH 130 100 $\mu\text{m} \times 100 \text{ mm}$ c18 separation column. A 30 min gradient (3% ACN to 40% CAN at 500 nL/min) after 3 min of trapping (99% water at 5 $\mu\text{L}/\text{min}$) was applied to separate peptides. MS was operated in V mode, acquiring MSE data and applying standard parameters. Data analysis was performed with protein lynx global server version 2.4 (Waters), searching an in house database consisting of the Uniprot database (May 2011, restricted to reviewed entries of eukaryotic organisms) and the amino acid sequences of recombinant and natural Sola l 4.0101 and recombinant and natural Sola l 4.0201. Protein hits were accepted at a false positive rate of less than 4%; peptide mass accuracy was 7 ppm or better. For the identification of natural Sola l 4 LC-MS was applied as described above. Briefly, protein extract from ripe tomatoes was separated by SDS-PAGE. All proteins between a molecular weight of 10 kDa and 45 kDa were excised and analyzed as described above.

2.4 Preparation of tomato extract

Tomato extract was prepared from fresh whole tomatoes of the variety *Verona* by direct homogenization of frozen tomato pieces in 100 mM ammonium carbonate buffer, pH 8.0 and incubation for 6 h at 4°C by end over end rotation. After centrifugation (30 min, 12 000 $\times g$, 4°C), the supernatant was precipitated with four volumes of ice-cold acetone over night at -20°C and centrifuged for 30 min (12 000 $\times g$, 4°C). The pellet containing the proteins was dried for 2 h in a fume hood and dissolved in PBS. Total protein content was quantified using the BCA protein assay (Fisher Scientific).

2.5 SDS-PAGE and immunoblotting

The purity of rSola l 4 isoforms was analyzed by SDS-PAGE (T: 15%, 2 $\mu\text{g}/\text{lane}$) [28] under reducing conditions and staining with GelCode Blue Stain reagent (Fisher Scientific). For immunoblotting, tomato extract (40 $\mu\text{g}/\text{cm}$) was subjected to SDS-PAGE under nonreducing conditions, transferred to a 0.2 μm nitrocellulose membrane (Protran, Whatman, Dassel, Germany) by semi-dry blotting [29] and stained with Ponceau S (Sigma-Aldrich, Munich, Germany). Detection of IgE binding was done as described elsewhere [30]. For inhibition of IgE-binding, serum samples (dilution 1:10) were incubated

with 10 μg or 50 μg (per 600 μL diluted serum) of rSola l 4 isoforms, rBet v 1 or, as negative control, BSA.

2.6 ELISA and IgE-inhibition assay

ELISA and IgE-inhibition experiments were performed as described elsewhere [30] with the slight modification that coating was done with 2.5 $\mu\text{g}/\text{mL}$ rSola l 4 isoforms, respectively.

2.7 Preparation of experimental ImmunoCAPs

Experimental ImmunoCAPTM testing was performed as described elsewhere [31].

2.8 Basophil histamine release

Histamine release with stripped and passively sensitized basophils was performed as described elsewhere [16].

3 Results

3.1 cDNA sequence analysis and characterization of Sola l 4

By using the RT-PCR approach we identified Bet v 1-like cDNA sequences (Genbank Acc. nos. KF682291 and KF682292) in tomato fruits from cultivar *Verona* confirming sequences obtained from a gene expression library from tomato cultivar *Micro-Tom* [32]. Although one identical Bet v 1-like cDNA sequence from tomato (GenBank Acc. no. AK247106) was described to occur only in leaf, we were able to amplify this cDNA also from fruits. The Bet v 1-like cDNA sequences, designated Sola l 4.0101 (GenBank Acc. no. KF682291) and Sola l 4.0201 (GenBank Acc. no. KF682292), both shown in detail in Table 2A, encoded proteins of 161 amino acids with a calculated MW of 18 kDa for Sola l 4.01 and 17.5 kDa for Sola l 4.02 and a pI of 5.35 and 5.44, respectively. The two Sola l 4 proteins share 81% amino acid identity (aa-id) with each other and are therefore considered isoforms. Sola l 4.01 and Sola l 4.02 show 44% and 42% aa-id to Bet v 1.0101 (Acc. No. X15877), respectively, and aa-id up to 52% with other Bet v 1-like food allergens (Table 2B). Sola l 4 isoforms were expressed as N-terminal (His)₆-tagged proteins of 182 aa with a calculated molecular weight of 20.3 kDa for Sola l 4.01 and 19.7 kDa for Sola l 4.02, and a pI of 5.1 (Fig. 1A). CD-spectroscopy showed identical secondary structures of the two isoforms, highly similar to Bet v 1.01 indicating all proteins were likely correctly folded (Fig. 1B). The MS data confirmed the identity of the recombinant tomato allergens. Eleven peptides were detected, covering approximately 50% of the amino acid sequence of rSola l 4.0101. The ProteinLynx Global SERVERTM (PLGS) score was 11 683 and the root-mean square error was 3.2 ppm. Recombinant Sola l 4.0201 was confirmed by

Table 2. (A) Amino acid sequences of the identified Sola l 4 isoforms. (B) Amino acid sequence identity of Sola l 4 isoforms with allergenic PR-10 proteins from food (Pru ar 1 from apricot, Mal d 1 from apple, Gly m 4 from soybean, Api g 1 from celeriac) and birch pollen (Bet v 1.01)

(A)

Sola l 4.0101 Acc. No. KF682291	M G V N T Y T Y E S T T T T I S P T R L F K A L V L D F D N L V P K 1 ATGGGTGTGAATACCTATACCTATGAGTCAACAACCAAAATTTCCCAACAAGACTATTCAAAGCTTTGGTTCTTGATTTTGACAACCTTGACCTAAA L L S Q H V K N N E T I E G D G G V G S I K Q M N F V E G G P I K 100 TTGTTGTCAACATGTTAAGAACAATGAGACTATTGAGGGAGATGGTGGTGTGAAGCATCAAGCAAATGAACCTTTGTTGAAGGTGGTCCAAATAAAG Y L K H K I H V I D D K N L E E T K Y S L I E G D I L G E K L E S I 199 TACTTGAACACAAGATTCTATGTGATTGATGACAAGAAGCTTAGAACAATAATTCATTATTGAAGGTGATTTCTTGGAGAAAAATGGAATCAATT T Y D I K F E A N D N G G C V Y K T T T E Y H T K G D H V V S E E 298 ACTTATGATATCAAATTTGAAGCTAATGATAATGGAGGTTGTGTTTACAAGACAACAAGTATGATACACAAGGGTGATCATGTTGTTAGTGAAGAA E H N V G R E R I M N I S K A V E A Y L L A N P S V Y A * I L M K 397 GAACACAATGTAGGCAGAGAGAATCATGAATATTTCCAAGGCTGTAGAAGCATACCTTCTCGCAATCTTCTGTCTACGCTTGAATATTGATGAAA K N Q V Y T * 496 AAGAATCAGGTCTACACGTGA
Sola l 4.0201 Acc. No. KF682292	M G V N T F T H E S T T T T I A P T R L F K G L V L D F D S L V P K 1 ATGGGTGTAAACACCTTTACTCATGAGTCCACAACCAAAATTTGCCCAACAAGGCTATTCAAAGGTTTGGTTCTTGATTTTGATAGCCTTGACCTAAA L L S H D V K S I E I V E G D G G A G S I K Q M N F V E G G P I K 100 TTGTTGTCAATGATGTTAAGAGCATGAGATTGTTGAGGGAGATGGTGGTGTGAAGCATCAAGCAAATGAACCTTTGTTGAAGGTGGACCAATTAAG Y L K H K I H V I D D K N L V T K Y S L I E G D V L G D K L E S I 199 TACTTGAAGACAAGATTCTATGTGATTGATGACAAGAATTTGGTAACAATAATTCATTATTGAAGGTGATGTTCTTGGTGAACAATGGAATCAATT A Y D V K F E A A G D G G C V C K T T T E Y H T K G D H V V S E E 298 GCCTATGATGTCAAATTTGAAGCTGCTGGAGATGGAGTTGTGTTTGAAGACAACAAGTATGATACACAAGGGTGATCATGTTGTTAGTGAAGAA E H N V G K G K A I D L F K A I E A Y L L A N P S V Y A * 397 GAACACAATGTAGGCAAGGGAAGCCATTGACCTATTCAAGGCCATCGAAGCGTACCTCTCGCTAATCTTCTGTCTACGCTTAA

(B)

	Sola l 4.01 KF682291	Pru ar 1 O50001	Mal d 1 AF124823	Gly m 4 X60043	Bet v 1.01 X15877	Api g 1.01 Z48967	Api g 1.02 Z75662
Sola l 4.02 KF682292	81	52	47	47	42	42	40
Sola l 4.01 KF682291		51	43	43	44	39	40
Pru ar 1 O50001			72	51	60	46	47
Mal d 1 AF124823				53	57	39	37
Gly m 4 X60043					47	39	38
Bet v 1.01 X15877						42	38
Api g 1.01 Z48967							52

detecting 7 peptides covering 38% of the amino acid sequence with a score of 5174 and a root-mean square error of 3.8 ppm.

3.2 Sola l 4 is a Bet v 1-homologous allergen in tomato fruits

Initial screening of sera from tomato allergic patients by indirect ELISA revealed a similar prevalence of IgE reactivity of 28% to rSola l 4.01 and rSola l 4.02 (data not shown). Subsequently, experimental ImmunoCAP™ testing was performed with rSola l 4.02 and rBet v 1.01 to quantify-specific IgE levels in sera from birch pollen allergic patients with (Fig. 2A) and without (Fig. 2B) tomato allergy. ImmunoCAP™ data revealed prevalence of specific IgE to Sola l 4 in 76% and to Bet v 1 in 81% of tomato allergic subjects. Three tomato allergic patients with reactivity to Bet v 1 were not sensitized to Sola l 4 and one patient showed reactivity to Sola l 4, but was not reactive with Bet v 1. Seven out of 42 sera tested, did not show IgE binding to any of the two PR-10 proteins. Notably, all birch pollen allergics without tomato allergy were sensitized to both Bet v 1 and Sola l 4. In both patient groups we observed a good correlation ($R^2 = 0.595$ and 0.432) between Bet v 1 and Sola l 4 specific IgE values (Fig. 2A, B). The correlation ($R^2 = 0.226$) between birch pollen and tomato specific IgE levels was considerably lower in tomato allergic patients (Fig. 2C). Statistically significant differences between

the groups were only observed for Bet v 1 specific IgE (median group 1: $19.05 \text{ kU}_A/\text{L}$, median group 2: $40.65 \text{ kU}_A/\text{L}$, $R^2: 0.099$, $p \text{ value} = 0.009$) (Fig. 3B). However, specific IgE concentrations to Sola l 4 in the two groups were not significantly different; Sola l 4: median group 1: $2.38 \text{ kU}_A/\text{L}$, median group 2: $11.85 \text{ kU}_A/\text{L}$, $R^2: 0.015$, $p \text{ value} = 0.313$ (Fig. 3A). In summary, according to the definitions of the IUIS Allergen Nomenclature Subcommittee, Sola l 4 qualifies as major allergen in tomato fruits. Sensitization to Bet v 1 was strongly associated with IgE-reactivity to Sola l 4 regardless whether patients tolerate or react to tomatoes.

3.3 Sola l 4 and Bet v 1 show strong IgE cross-reactivity

Cross-reactivity of IgE between Sola l 4 isoforms and Bet v 1 was evaluated by competitive ELISA experiments using Sola l 4-reactive sera from 11 patients (two samples are depicted in Fig. 4). In the experimental setting Sola l 4 isoforms were used on the solid phase, and Bet v 1 as well as the tomato proteins were used as inhibitors. Sola l 4.02 showed stronger (e.g. serum no. 05 + 31) or at least similar (e.g. serum no. 60, data not shown) inhibition capacity as Sola l 4.01, depending on the individual serum tested. Noteworthy, for the majority of sera tested, Bet v 1 was a more efficient inhibitor of IgE binding to the Sola l 4 isoforms than these proteins

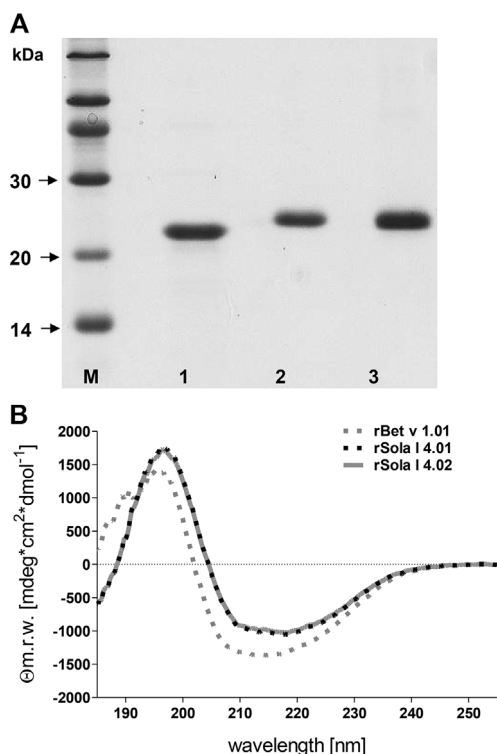


Figure 1. Purification and structural characterization of Sola l 4 isoforms. (A) SDS-PAGE analysis and Coomassie staining of rBet v 1.01 (lane 1), rSola l 4.02 (lane 2), and rSola l 4.01 (lane 3), molecular marker (M). (B) Circular dichroism (CD) spectroscopy of rSola l 4.01 and rSola l 4.02 in comparison to rBet v 1.01.

themselves (e.g. serum no. 05). Here, Bet v 1 showed up to 300-fold stronger IgE binding than Sola l 4. Only for a limited number of sera we observed lower capacity of Bet v 1 to compete IgE binding to the tomato homologue Sola l 4 (e.g. serum no. 31). Interestingly, the capacity to inhibit IgE binding to Sola l 4 and Bet v 1 varied among individual sera tested but no correlation to the donors' allergy or tolerance to tomato was found.

3.4 Sola l 4 induces mediator release from passively sensitized peripheral blood mononuclear cells

Allergenic potency of Sola l 4 was evaluated by in vitro histamine release tests using Sola l 4-reactive sera from 13 patients and the passive sensitization technique. Sola l 4 was able to induce substantial histamine release (>10% cut-off) using sera from 4/5 tomato allergic patients, but only in 2/8 tomato tolerant patients. Representative mediator release curves for two tomato allergic (nos. 05 + 31) and two tomato tolerant (nos. 55 + 63) patients are depicted in Fig. 5 (sera are indicated in bold in Table 1). Likewise, Sola l 4.02 showed stronger (e.g. serum no. 63) or at least similar (e.g. serum no. 31) allergenic potency as Sola l 4.01, depending on the individual serum tested. Only in one patient Sola l 4.01 was able

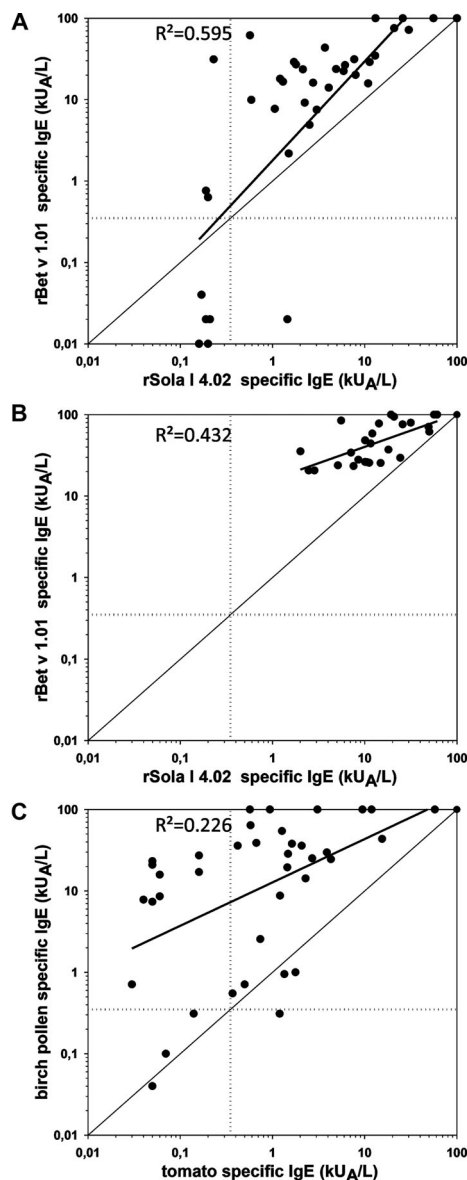


Figure 2. Determination of rSola l 4.02, rBet v 1.01, tomato, and birch pollen-specific IgE values by means of experimental ImmunoCAP™ testing. Comparison of rSola l 4.02 and rBet v 1.01-specific IgE using sera from birch pollen allergic patients (A) with clinical history of allergy ($n = 42$) or (B) tolerance ($n = 26$) to tomato. (C) Comparison of tomato and birch pollen-specific IgE in birch pollen allergic patients with tomato allergy.

to trigger stronger mediator release than Sola l 4.02 (no. 48, data not shown). For almost all sera tested, Bet v 1 showed the strongest allergenic potency (e.g. serum nos. 05, 55 + 63). For a limited number of samples (e.g. serum no. 31), the allergenic potency of Bet v 1 and Sola l 4 were comparable. Noteworthy, the low frequency of sera from tomato tolerant patients responding to Sola l 4 by mediator release was not associated with significantly increased total IgE values

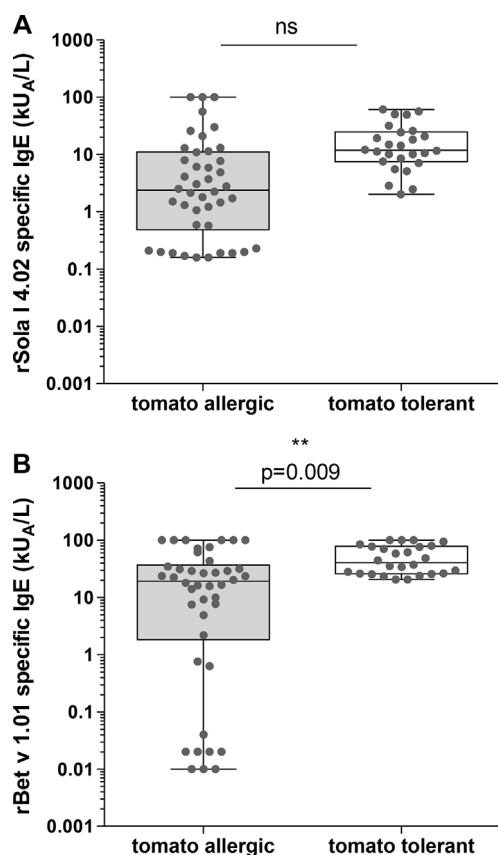


Figure 3. Comparison of tomato allergic patients ($n = 42$) and tomato tolerant patients ($n = 26$) in terms of specific IgE to (A) rSola l 4.02 and (B) rBet v 1.01 by means of experimental ImmunoCAPTM testing.

compared to sera from tomato allergic patients (total IgE: median group 1: 485 kU_A/L, median group 2: 405.5 kU_A/L, R^2 : 0.052, p value = 0.218).

3.5 Detection of a Bet v 1 homologous protein in tomato fruits

To further confirm the expression of natural Sola l 4 in tomato fruits, immunoblot inhibition experiments were performed, using both recombinant Sola l 4 isoforms and rBet v 1 as inhibitors. A Sola l 4 corresponding protein with an apparent molecular weight of 20 kDa was barely detectable in different tomato cultivars, by probing different Sola l 4 reactive sera and PR-10 reactive monoclonal antibodies, as well as using various protein extraction protocols and detection methods (data not shown). Nevertheless, both rSola l 4 isoforms and rBet v 1 were able to outcompete IgE binding to a protein with an apparent molecular weight of 20 kDa in tomato fruits of the cv *Verona* (Fig. 6), likely the cross-reactive homologous Sola l 4 in tomato fruits. Furthermore, natural Sola l 4.02 was detected by LC-MS analyses of protein extracts of tomato fruits. Seven peptides covering 44% of the amino acid se-

quence of Sola l 4.02 (Acc. no. K4CWC4, which is equivalent to Acc. no. AK224718 as none of the previously used Acc. nos. is present in the UniProt derived In-house database) were detected with a score of 748 and a RMS mass error of 2.3 ppm.

4 Discussion

Tomato allergy has been suggested to be clinically associated with birch pollen allergy [6]. If patients with concomitant birch pollen allergy are excluded, the prevalence of tomato sensitization decreases from 3.3 to 2.3% of food allergic subjects [2]. Furthermore, a recent Italian study showed 20 of 32 tomato allergic patients to be sensitized to the major birch allergen Bet v 1 [4]. These findings support the cross-reactivity of IgE to birch pollen with tomato allergens and provide indirect evidence for the presence of a Bet v 1 homologous allergen in tomato fruits. Even though tomato allergic patients display sensitization to multiple tomato proteins, until now no Bet v 1 homologous protein has been described as tomato allergen. Therefore, this study aimed at the identification and investigation of the allergenic properties and clinical relevance of a Bet v 1 homologous protein in tomato.

Utilizing the tomato sequence databank MiBASE, we cloned and purified two recombinant PR-10 proteins from tomato cv *Verona*, which have been accepted by the IUIS Allergen Nomenclature Subcommittee as Sola l 4.01 and Sola l 4.02. Noteworthy, a Bet v 1-like cDNA sequence published by Sree Vidya et al. [33] that is almost identical to Sola l 4.01 shows one base pair insertion at position 415, which leads to a frameshift and thereby to an extended amino acid sequence of 18 amino acids affecting the structural integrity and showing diminished IgE-reactivity (data not shown). In the present study we provided evidence that both Sola l 4 isoforms are correctly folded and immunologically reactive. Sola l 4.02 was selected for single component analysis by experimental ImmunoCAPTM testing. Results showed IgE binding to Sola l 4 to be strongly associated with IgE reactivity to Bet v 1, even in patients without tomato allergy indicating a high sensitivity but low specificity using Sola l 4 for component-resolved diagnosis in tomato allergic patients. Notably, we observed higher Bet v 1-specific IgE levels in birch pollen allergic patients without tomato allergy (group 2) than in patients with tomato allergy (group 1). This may be explained by the fact that patients in group 1 were selected by clinical history of tomato allergy and patients in group 2 were selected by birch pollen allergy and concomitant sensitization to Bet v 1. Only one tomato allergic patient was sensitized to Sola l 4 but not reactive to Bet v 1 and vice versa, three sera were reactive to Bet v 1 but not to Sola l 4. Taken together, the level of amino acid identity over 40%, similar protein structures and covariation in IgE binding, suggested substantial IgE cross-reactivity between Sola l 4 and Bet v 1. Competitive IgE-binding assays confirmed cross-reactivity between these PR-10 proteins and Bet v 1 to be the strongest inhibitor,

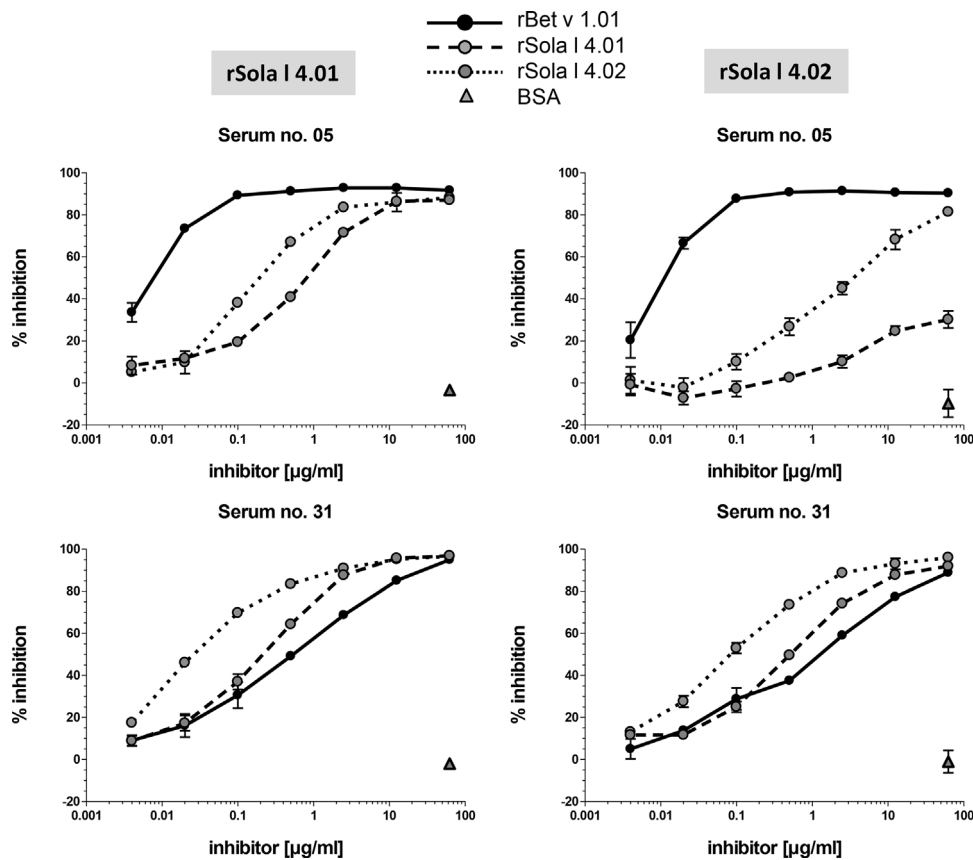


Figure 4. IgE cross-inhibition between Sola I 4 isoforms and Bet v 1. Sera from tomato allergic patients were preincubated dose dependently with the respective allergens. Competition of IgE-binding to rSola I 4.01 and rSola I 4.02 was measured by ELISA.

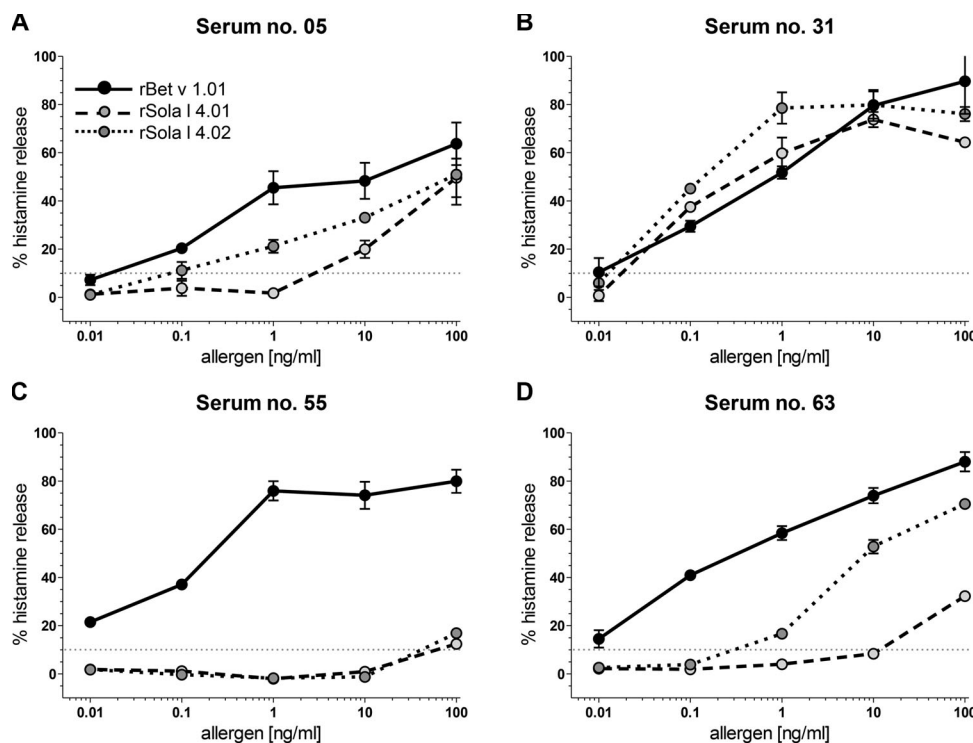


Figure 5. Determination of allergenic potency of Sola I 4 isoforms in comparison to Bet v 1 by means of in vitro histamine release assay using sera from (A, B) tomato allergic and (C, D) tomato tolerant patients.

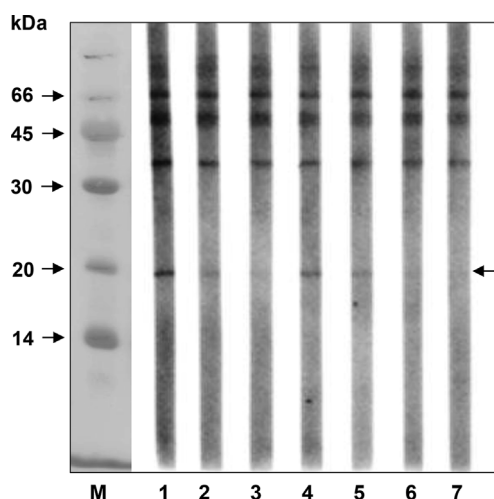


Figure 6. Detection of Sola l 4 expression in tomato fruits. IgE binding to tomato extract (cv *Verona*) was inhibited by preincubation of a Sola l 4-reactive serum with rSola l 4.02 (10 and 50 $\mu\text{g}/\text{mL}$, lanes 2 + 3), rSola l 4.01 (10 and 50 $\mu\text{g}/\text{mL}$, lanes 4 + 5), and rBet v 1.01 (10 and 50 $\mu\text{g}/\text{mL}$, lanes 6 + 7). M: Molecular marker; no inhibitor (lane 1).

suggesting primary sensitization to birch pollen rather than to tomato. Nevertheless, for a limited number of sera, Bet v 1 competed less efficiently for IgE binding to Sola l 4.02 than did Sola l 4.02 itself. Further, IgE binding to Sola l 4.01 was more efficiently inhibited by Sola l 4.02 than IgE binding to Sola l 4.02 by Sola l 4.01. In addition to demonstrating cross-reactivity between PR-10 proteins from birch and tomato, the results also indicated the diverse IgE-epitope recognition of the Sola l 4 isoforms among the individual sera tested. Both Sola l 4 isoforms displayed allergenic activity by triggering mediator release upon stimulation of passively sensitized basophils. In general, the allergenic potency was strongest for Bet v 1, moderate for Sola l 4.02 and slightly weaker for Sola l 4.01. Of note, we observed a lower number of tomato tolerant patients than tomato allergic patients responding to Sola l 4 by histamine release, all with a level of IgE to Sola l 4 corresponding to ImmunoCAPTM class 3 or higher. To establish whether this finding is statistically significant, sera of a larger number of subjects need to be analyzed. Remarkably, the induction of mediator release by Sola l 4 was not affected by the concentration of total IgE that might be considered to bind to $\text{Fc}_\epsilon\text{RI}$ and block binding of lower amounts of Sola l 4-specific IgE.

Despite the capacity of Sola l 4 to induce mediator release, clinical relevance has not been directly proven and likely depends on the amount of Sola l 4 present in tomato fruit. In our study, high Sola l 4-specific IgE-levels were not associated with high IgE-levels to tomato extract, indicating that the allergen may not be abundantly represented in tomato extracts, most likely due to a low expression level in tomato fruits. These results are in line with Asero et al. [4] who observed no correlation between birch pollen-specific and tomato-specific

IgE-levels in patients mono-sensitized to Bet v 1. Low expression of Sola l 4 in tomato fruits among other IgE-reactive proteins is substantiated by a faint IgE binding to Sola l 4 in immunoblot inhibition analysis of protein extracts from cv *Verona*. Moreover, mass spectrometric analysis clearly confirmed the presence of one of the Sola l 4 isoforms in tomato fruit extract. However, the expression level of the tomato PR-10 allergen in other tomato varieties and potential up-regulation upon biotic and abiotic stress need to be further investigated.

In summary, two isoforms of a Bet v 1-homologous protein, designated as Sola l 4, were identified in tomato fruits. Sola l 4.02 showed strongest immunological activities and was characterized as major allergen in tomato fruits. In vitro diagnosis using Sola l 4 showed high sensitivity but low clinical specificity. Further conclusions about the clinical relevance of Sola l 4 needs to consider the level of accumulation of the allergen in tomato fruits and symptom eliciting threshold levels in patients.

The authors thank Jens Dorna, Michaela Gubesch, Renate Hoepfner, Luisa Schwaben, Klaus Thalheim and Sonja Wolfheimer for excellent technical assistance. The study was funded by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG VI 165/6 and DFG MA 1997/3-1).

Potential conflict of interest statement: Jonas Lidholm is an employee of Thermo Fisher Scientific, manufacturer of ImmunoCAP IgE antibody tests. The following activities of Stefan Vieths could be perceived as a potential conflict of interest concerning this manuscript: In the past five years SV has received speakers honorarium from Phadia (now ThermoFisher Scientific), Uppsala, a honorarium for organising and chairing an educational seminar on food allergens from Fresenius Academy, Dortmund, honoraria for acting as expert reviewer for the Allergen Online Database of the Food Allergy Resource and Research Program, Lincoln NE, and consultancy fees by the Institute for Product Quality, Berlin. The other authors have declared no conflict of interest.

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