

Identification of a Dau c PRPlike protein (Dau c 1.03) as a new allergenic isoform in carrots (cultivar *Rodelika*)

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Abstract

Background Up to 25% of food allergic subjects in central Europe suffer from carrot allergy. Until now, two isoforms of the major carrot (*Daucus carota*) allergen Dau c 1 have been described: Dau c 1.01, comprising five variants (Dau c 1.0101–Dau c 1.0105) and Dau c 1.02.

Objective To investigate potential allergenic properties of a Dau c PRPlike protein, a novel isoform of the PR-10 protein family in carrot.

Methods Dau c PRPlike cDNA from carrot roots (cv *Rodelika*) was cloned after RT-PCR and 5'RACE. Dau c PRPlike protein was expressed in *E. coli*, purified under native conditions by Ni-NTA chromatography and analysed by CD spectroscopy. Immuno-reactivity of the rDau c PRPlike protein was compared with rDau c 1.0104 and rDau c 1.0201 in terms of IgE binding (immunoblotting, ImmunoCAPTM), IgE cross-reactivity (ELISA inhibition) and *in vitro* mediator release with sera from carrot allergic patients. mRNA expression of Dau c PRPlike protein in wild-type and transgenic carrot roots was analysed by qRT-PCR.

Results The Dau c PRPlike protein was identified as a new allergenic isoform, Dau c 1.03, in carrot roots. 68% of carrot allergic patients were sensitized to rDau c 1.03. The IgE-reactivity of rDau c 1.03 strongly correlated with reactivity to rDau c 1.0104, but not to rDau c 1.0201. The extent of IgE cross-reactivity and allergenic potency of Dau c 1 isoforms varied between the individual sera tested. Dau c 1.03 mRNA transcripts were up-regulated in Dau c 1.01 and Dau c 1.02 gene-silenced carrot roots.

Conclusion and clinical relevance Dau c 1 isoforms display distinct IgE epitope heterogeneity. Dau c 1.03 appears to contribute to the allergenicity of carrots and the manifestation of carrot allergy. The epitope diversity of different Dau c 1 isoforms should be considered for component-resolved diagnosis and gene silencing of carrot allergens.

Keywords carrot allergy, Dau c 1, IgE-epitopes, isoforms, PRPlike, recombinant allergen
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Introduction

Carrot (*Daucus carota*) allergy is one of the most common types of birch pollen-related food allergy in central Europe [1]. Approximately 24% of food allergic subjects suffer from allergic symptoms after ingestion of carrots [2, 3]. Adverse reactions to carrots are elicited due to cross-reactive IgE-epitopes between the major birch pollen allergen, Bet v 1 [4] and homologous food proteins [5–7]. Bet v 1 and the major carrot allergen Dau c 1 [7, 8] belong to the family of pathogenesis-related proteins 10 (PR-10).

So far, 30 isoallergens of Bet v 1 have been recognized by the IUIS Allergen Nomenclature Subcommittee. In contrast, only two isoforms (isoallergens) of the major carrot allergen, displaying as low as 50% amino acid sequence identity, have been described: Dau c 1.01 [8], comprising five variants (with amino acid identities $\geq 97\%$), Dau c 1.0101–Dau c 1.0105, and Dau c 1.02 [9]. Furthermore, other PR-10-like proteins from carrot, so-called Dau c PRPlike proteins, are described in the NCBI GenBank database, but have not yet been identified as allergens. In comparison to Dau c 1.02, Dau c 1.0104 is described as the isoform with the strongest

IgE-binding capacity [9]. Likely, Dau c 1 isoforms display heterogeneous IgE-binding sites. The presence of isoforms with different IgE-binding epitopes is of major concern for component-resolved diagnosis (CRD) using recombinant allergens. Ballmer-Weber et al. [9] described improved specificity and sensitivity of *in vitro* diagnosis of carrot allergy by using recombinant allergen molecules and demonstrated a good correlation between IgE to Dau c 1.02 and carrot allergy. However, when multiple allergen isoforms with different IgE-binding properties are present, a more comprehensive repertoire of isoforms may be required to achieve the highest possible diagnostic sensitivity. In addition, knowledge about clinically relevant isoforms is necessary to select target molecules for RNA interference (RNAi) mediated gene silencing to suppress allergen expression in fruits and vegetables [10–12]. Recently, highly specific small interfering RNA (siRNA) has been applied to selectively down-regulate certain Dau c 1 isoforms, as described for allergen suppression in transgenic carrots [11].

In the present study, IgE-binding properties, allergenic potency and cross-reactivity of a new Dau c PRP-like protein from carrot (cv *Rodelika*), denominated as Dau c 1.03, were compared with allergenic Dau c 1 isoforms, Dau c 1.01 and Dau c 1.02. Furthermore, Dau c 1.03 mRNA expression was analysed in wild-type and transgenic carrot roots. Our data indicate that the protein seems to be a relevant allergen in carrot and is not suppressed in the existing Dau c 1-silenced carrot variety.

Methods

Patients, patients' sera and ImmunoCAPTM testing

Forty carrot allergic patients, selected by positive case history of carrot allergy were included in the study (Table 1). OAS was the most frequently (78%, $n = 31$) observed symptom (whereas 15 patients reported systemic reactions upon ingestion of carrots). Sensitization to carrot was tested by skin prick testing (Bencard, Munich, Germany, carrot 599a/89N) or prick-to-prick testing (carrot cv *Rodelika*) in 35 of 40 patients. Levels of specific IgE to carrot extract (f31), recombinant (r) Bet v 1 and bromelain were determined by ImmunoCAPTM analysis (Phadia AB, Uppsala, Sweden) in all sera. IgE antibody concentrations of 0.35 kU_A/L or greater were considered positive. Experimental ImmunoCAPTM tests were generated with rDau c 1.0104, rDau c 1.0201 and rDau c PRPlike protein (referred to as Dau c 1.03 in the following) as described previously [13]. Carrot allergy was confirmed by positive reaction in open food challenge with raw carrots in all patients ($n = 14$) tested. Approval by the local ethics committee

(Faculty of Medicine, University of Erlangen-Nuremberg, No. 3494) for this study and written informed consent of all participants were obtained.

cDNA cloning, expression and purification of Dau c 1.03 protein

Total cDNA of *Rodelika*, a commercially available carrot variety, was prepared using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and First-Strand cDNA Synthesis Kit (GE Healthcare, Freiburg, Germany) according to the manufacturer's instructions. For polymerase chain reaction (PCR) based-amplification of Dau c PRP-like cDNA, degenerated primers 5'-ATG GGT GTC CAG AAG CAC GAA CAA G-3' (forward) and 5'-TTA RTT WGC WAK RAG RTA RGC YTC RAY RGC YTT GAA-3' (reverse) were designed using the Dau c PRPlike cDNA sequences (GenBank accession nos.: AB082377, AB127960, AB127984). Full-length cDNA was obtained by 3'RACE and 5'RACE (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Subsequently, one selected Dau c PRPlike cDNA (clone FL3, Dau c 1.03) was cloned into the expression vector pET23b (Novagen, Darmstadt, Germany). The Dau c 1.03/pET23b construct was transformed into BL21(DE3) competent *E.coli* (Novagen) and protein synthesis was induced with 1 mM isopropyl- β -D-thio-galactopyranoside (IPTG) overnight at 23°C at an optical density OD_{600 nm} = 0.6–0.8. Purification of the (His)₆-tagged Dau c 1.03 was performed by immobilized metal affinity chromatography (IMAC) with Ni-NTA under native conditions using a His GraviTrap column (GE Healthcare) according to the manufacturer's instructions. Recombinant (r) Dau c 1.0104 and rDau c 1.0201 were prepared as published elsewhere [9].

Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy of rDau c 1 isoforms 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 spectral 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 signal-to-noise ratio, 10 scans were accumulated. Measurements were performed in a 1 mm path length quartz cell (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 mdeg, c the protein concentration in mM, d

Table 1. Carrot allergic patients. Reported symptoms upon ingestion of carrot, open food challenge and skin prick test results; specific IgE values to carrot extract, rBet v 1, bromelain and rDau c 1 isoforms are indicated. Sera used for IgE-inhibition and histamine release assays are marked in bold.

No.	Clinical symptoms to carrot	Provocation	Skin test	CAP [kU _A /L] (CAP class)					
							rDau c	rDau c	rDau c
Patient	Symptoms	OFC	Carrot	Carrot (f31)	rBet v 1	Bromelain	1.0104	1.0201	1.0301
1	U	nd	pos	0.41 (1)	0.00 (0)	0.71 (2)	0.03 (0)	0.01 (0)	0.00 (0)
2	OAS (mild)	nd	pos	0.52 (1)	0.06 (0)	0.14 (0)	0.02 (0)	0.01 (0)	0.00 (0)
3	U	nd	pos	1.33 (2)	23.60 (4)	0.05 (0)	7.23 (3)	3.74 (3)	9.10 (3)
4	OAS, CU	pos	pos	0.10 (0)	6.62 (3)	0.03 (0)	0.00 (0)	2.04 (2)	0.04 (0)
5	OAS	nd	pos	2.76 (2)	>100 (6)	0.06 (0)	5.82 (3)	17.0 (3)	6.13 (3)
6	GI	nd	pos	0.00 (0)	0.00 (0)	0.02 (0)	0.03 (0)	0.01 (0)	0.00 (0)
7	OAS (mild)	nd	pos	7.98 (3)	0.09 (0)	8.31 (3)	0.06 (0)	0.09 (0)	0.01 (0)
8	OAS (mild)	nd	pos	1.06 (2)	>100 (6)	0.82 (2)	8.45 (3)	12.00 (3)	6.30 (3)
9	OAS (mild)	pos	pos	0.07 (0)	43.10 (4)	0.28 (0)	5.25 (3)	4.32 (3)	9.75 (3)
10	OAS	pos	pos	2.82 (2)	35.9 (4)	0.88 (2)	3.70 (3)	4.32 (3)	3.57 (3)
11	OAS (mild)	nd	pos	0.19 (0)	13.00 (3)	0.34 (0)	1.01 (2)	0.04 (0)	0.87 (2)
12	OAS	nd	pos	0.02 (0)	0.05 (0)	0.08 (0)	0.02 (0)	0.03 (0)	0.01 (0)
13	OAS	nd	pos	0.00 (0)	0.00 (0)	0.01 (0)	0.00 (0)	0.01 (0)	0.00 (0)
14	OAS, Q	nd	pos	0.79 (2)	7.56 (3)	0.02 (0)	1.17 (2)	1.43 (2)	1.38 (2)
15	U, D	pos	pos	0.71 (2)	2.58 (2)	0.63 (1)	0.02 (0)	0.17 (0)	0.09 (0)
16	Q	nd	pos	0.00 (0)	0.00 (0)	0.02 (0)	0.01 (0)	0.02 (0)	0.00 (0)
17	OAS	pos	pos	5.20 (3)	55.80 (5)	0.18 (0)	10.50 (3)	4.90 (3)	7.93 (3)
18	Q, D, U	nd	nd	0.05 (0)	7.14 (3)	0.02 (0)	0.85 (2)	0.20 (0)	0.67 (1)
19	GI, Q	nd	neg	0.25 (0)	16.60 (3)	0.11 (0)	0.82 (2)	0.40 (1)	3.12 (2)
20	OAS	pos	pos	2.20 (2)	9.28 (3)	0.02 (0)	2.53 (2)	1.55 (2)	2.61 (2)
21	OAS	pos	pos	2.51 (2)	11.90 (3)	0.21 (0)	1.95 (2)	0.77 (2)	0.92 (2)
22	OAS	pos	pos	0.62 (1)	5.35 (3)	0.08 (0)	0.64 (1)	0.91 (2)	0.73 (2)
23	OAS, D	pos	pos	0.44 (1)	3.16 (2)	0.03 (0)	0.59 (1)	0.45 (1)	0.48 (1)
24	Q, D	nd	neg	0.37 (1)	69.80 (5)	0.24 (0)	0.71 (2)	2.15 (2)	2.29 (2)
25	OAS (mild)	nd	pos	0.02 (0)	>100 (6)	0.25 (0)	0.02 (0)	3.03 (2)	0.09 (0)
26	OAS	nd	pos	1.43 (2)	>100 (6)	0.21 (0)	12.00 (3)	0.77 (2)	8.57 (3)
27	OAS	nd	pos	0.34 (0)	6.91 (3)	0.18 (0)	1.73 (2)	0.21 (0)	1.31 (2)
28	GI, OAS	pos	pos	0.00 (0)	0.02 (0)	0.06 (0)	0.03 (0)	0.02 (0)	0.01 (0)
29	OAS, D	nd	pos	0.68 (1)	7.39 (3)	0.02 (0)	1.50 (2)	1.21 (2)	0.94 (2)
30	OAS	pos	pos	1.05 (2)	10.50 (3)	0.07 (0)	0.69 (1)	1.06 (2)	1.06 (2)
31	OAS	nd	nd	0.13 (0)	0.00 (0)	0.10 (0)	0.00 (0)	0.01 (0)	0.03 (0)
32	OAS, GI	pos	neg	0.42 (1)	29.40 (4)	0.17 (0)	1.29 (2)	2.28 (2)	1.28 (2)
33	OAS	nd	pos	0.93 (2)	0.06 (0)	0.88 (2)	0.02 (0)	0.03 (0)	0.03 (0)
34	GI (raw)	nd	pos	11.40 (3)	0.64 (1)	12.40 (3)	0.27 (0)	0.34 (0)	0.40 (1)
35	OAS	pos	pos	24.00 (4)	>100 (6)	2.90 (2)	28.40 (4)	16.60 (3)	28.40 (4)
36	OAS	nd	pos	0.61 (1)	6.32 (3)	0.06 (0)	0.58 (1)	0.47 (1)	0.76 (2)
37	OAS	pos	pos	8.16 (3)	0.06 (0)	0.36 (1)	4.86 (3)	14.40 (3)	6.92 (3)
38	OAS (raw)	nd	nd	9.64 (3)	37.5 (4)	0.89 (2)	11.60 (3)	5.02 (3)	17.80 (4)
39	OAS (raw)	nd	nd	4.21 (3)	66.80 (4)	0.02 (0)	6.95 (3)	1.59 (2)	7.86 (3)
40	OAS	nd	nd	9.23 (3)	71.80 (5)	nd	11.26 (3)	11.30 (3)	18.30 (4)

OAS, Oral allergy syndrome; U, urticaria; CU, contact urticaria; GI, gastrointestinal symptoms; Q, quinke symptoms; D, dyspnea; nd, not determined.

the light path in cm and *N* is the number of amino acid residues.

Preparation of carrot extract

Carrot extract was prepared from fresh carrot roots of the variety *Rodelika* by a low-temperature method, as previously described [14, 15]. Measurement of total pro-

tein content was done with the Roti[®]-Nanoquant solution (Roth, Karlsruhe, Germany) according to the method of Bradford [16].

SDS-PAGE and IgE-immunoblotting

Purity of the recombinant proteins and protein pattern of carrot extract were monitored by SDS-PAGE (T:15%)

[17] with a protein loading of 1.7 and 20 µg per cm of gel width, respectively, and staining with GelCode Blue Stain Reagent (Thermo Scientific, Nidderau, Germany). For immunoblotting, recombinant proteins (0.5 µg/cm) and carrot extract (20 µg/cm) were subsequently transferred to a 0.2 µm nitrocellulose membrane (Protran, Whatman, Dassel, Germany) by semi dry blotting [18] and stained with Ponceau S (Sigma–Aldrich, Munich, Germany). Detection of bound human IgE from sera of carrot allergic patients was done as described elsewhere [19] with slight modifications as follows: The membrane was blocked with 2% skimmed milk in Tris-buffered saline (TBS-M), sera were diluted 1 : 7.5 in TBS supplemented with 0.05% Tween20 and 2% skimmed milk (TBST-M) and bound IgE was detected using an HRP-labelled mouse anti-human IgE antibody (Southern Biotech, Birmingham, AL, USA) diluted 1 : 10 000 in TBST-M.

IgE-inhibition by ELISA

For ELISA inhibition experiments 0.5 µg/mL rDau c 1.0104, 5 µg/mL rDau c 1.0201 and 4 µg/mL rDau c 1.03 (100 µL/well, in 50 mM Na₂CO₃/NaHCO₃, pH 9.6) were coated overnight at 4°C to Maxisorp™ plates (96 wells) (Nunc, Wiesbaden, Germany) and blocked with PBS supplemented with 0.05% Tween20 and 2% BSA (PBST-BSA, 250 µL/well) for 1 h at RT. Sera (50 µL/well) were diluted in PBST-BSA to reach an OD_{450 nm} between 0.6 and 0.8 and incubated overnight at RT with serial dilutions of rDau c 1 isoforms and carrot extract as inhibitor (50 µL/well). Bound specific IgE was detected with 100 µL/well of HRP-labelled mouse anti-human IgE (Southern Biotech) diluted 1 : 5000 in PBST-BSA by 2 h incubation at RT. 3,3',5,5'-Tetramethylbenzidine (TMB) supplemented with 0.01% H₂O₂ (in 0.21 M potassium citrate buffer, pH 3.95, 100 µL/well) was used as substrate. Colour reaction was stopped with 25% sulphuric acid (50 µL/well). OD was determined bichromatically at 450/630 nm in a SpectraMax 340 microplate reader (Molecular Devices, Ismaning, Germany). The percentage of IgE-inhibition was calculated as follows (B: serum with inhibitor; B0: serum without inhibitor; NSB: non-specific binding of immuno reagents):

$$\text{Inhibition(\%)} = \frac{[\text{OD(B0)} - \text{OD(B)}] / [\text{OD(B0)} - \text{OD(NSB)}] \times 100.}$$

Basophil histamine release

Histamine release with stripped and passively sensitized basophils was performed as described by Kleine-Budde et al. [20] with slight modifications [21].

Structure modelling and calculation of conserved surface residues

A model of the tertiary structure of Dau c 1.0104 was generated by Swiss-Model [22–24] using Dau c 1.0103 (PDB accession number: 2WQL) as template. Determination of the conserved surface residues was performed as described by Jenkins et al. [25] using T-Coffee [26] for sequence alignment and ViewerLite 5.0 (Accelrys, San Diego, CA, USA) for modelling.

Determination of Dau c 1.03 mRNA transcripts

Dau c 1.03 mRNA transcripts from cv *Rodelika* wild-type and transgenic carrot roots [11], either infected by *Alternaria radicina* (10 µL aqueous suspension containing 10⁵ conidia per root slice) or mock treated, were quantified by qRT-PCR. RNA was isolated from homogenized carrot roots by TRIZOL Reagent (Invitrogen) as described by the manufacturer. Total RNA (1 µg) was used for cDNA synthesis with a QuantiTect RT-Kit (Qiagen). Aliquots of 7.5 ng cDNA from three pooled replicates were used as template. An 147-bp cDNA fragment of Dau c 1.0301 was amplified by means of qRT-PCR (Applied Biosystems 7500 Fast Real Time PCR, Applied Biosystems Inc., CA, USA) using Dau c 1.0301 specific primers: 5'-TGT CCA GAA GCA CGA ACA AG-3' (forward) and 5'-CCA ACT CCA CCA TCA CCT TT-3' (reverse). Isoform specificity of oligonucleotides was assessed by RT-PCR (annealing temperature 60°C, 40 cycles) and sequencing of amplified cDNA from wild-type carrots, as well as by RT-PCR using Dau c 1.0104/pET16b, Dau c 1.0201/pET16b and Dau c 1.0301/pET23b constructs (5 ng each) as templates. Moreover, a 169-bp cDNA fragment of constitutively expressed actin (GenBank accession no. X17525) was amplified using actin specific primers: 5'-CCA CTG AAC CCA AAAGCA A-3' (forward) and 5'-CCG TGT GGC TAA CAC CAT C-3' (reverse).

Results

Cloning of Dau c 1.03 cDNA and sequence analysis

By using the RT-PCR approach, we identified seven new Dau c PRPlike cDNA sequences (GenBank accession nos.: HM064421–7) in carrot roots (cv *Rodelika*). The deduced Dau c PRPlike proteins shared 94–99% amino acid identity (aa-id) and all belonged to the PR-10 protein family. All new Dau c PRPlike proteins can be further sub-classified as DcPRPlike 1 proteins with an aa-id up to 99% to DcPRPlike 1 (GenBank accession no. AB082377). In contrast, Dau c PRPlike proteins displayed 80–82% aa-id with DcPRPlike 2 protein (GenBank accession no. AB127960). Dau c PRPlike

protein HM064421 (clone FL3, Dau c 1.03) showed the lowest aa-id to Dau c 1.0104 (aa-id 70%) and Dau c 1.0201 (aa-id 48%), and was selected for comparison with known Dau c 1 allergens. Dau c 1.03 consists of 155 amino acids and has a deduced molecular weight of 16.6 kDa and a theoretical pI of 5.11.

Identification of Dau c 1.03 as an allergenic isoform of the major carrot allergen

Recombinant (r) Dau c 1.0104, rDau c 1.0201 and rDau c 1.03 were expressed in *E. coli* and purified using IMAC (Fig. 1a). Under non-reducing condition rDau c 1.0201 displayed an additional weak band with an apparent molecular weight of 40 kDa, which could be attributed to a low degree of dimerization as frequently observed upon sample preparation for SDS-PAGE analysis (data not shown). CD spectroscopy indicated all isoforms to be folded, displaying a comparable content of secondary structures among Dau c 1.0301 and Dau c 1.0104. The CD spectrum of Dau c 1.0201 was slightly shifted, which likely is attributed to a different content of secondary structure elements between Dau c 1.0201 and Dau c 1.0104. Corresponding results have been described by Ballmer-Weber et al. [9] and also been found for the homologous allergen from Api g 1.02 from celery [27]. Screening of IgE-binding to rDau c 1.0104, rDau c 1.0201, rDau c 1.03 and carrot extract was performed with 23 sera by immunoblotting (data not shown). While 83% (19/23) of the sera showed IgE binding to carrot extract, reactivity to natural Dau c 1 (attributed to a protein with an apparent molecular weight of 18 kDa in carrot extract) was detected in 57% (13/23) of the same sera. Applying recombinant Dau c 1 isoforms, immunoblotting results showed sensitization to rDau c 1.0104 and rDau c 1.0201 in 70% (16/23) of the patients, and to rDau c 1.03 in 57% (13/23) (data not shown). IgE-reactivity to recombinant isoforms is illustrated in Fig. 1c with selected sera from carrot allergic patients nos. 9, 35, and 40 (Table 1). In addition, a slight formation of dimers of Dau c 1.0104 and Dau c 1.0201 is indicated by SDS-PAGE and immunoblotting. Hence, Dau c 1.03 has been confirmed as new allergenic isoform and was denominated as Dau c 1.0301 by the IUIS Allergen Nomenclature Subcommittee.

Determination of Dau c 1 isoform specific IgE by ImmunoCAP

To determine concentrations of IgE antibodies to the Dau c 1 isoforms, experimental ImmunoCAPTM tests were performed on all carrot allergic patients ($n = 40$) included in the study (Table 1). 68% (27/40) of the sera reacted with carrot extract. Using the recombinant iso-

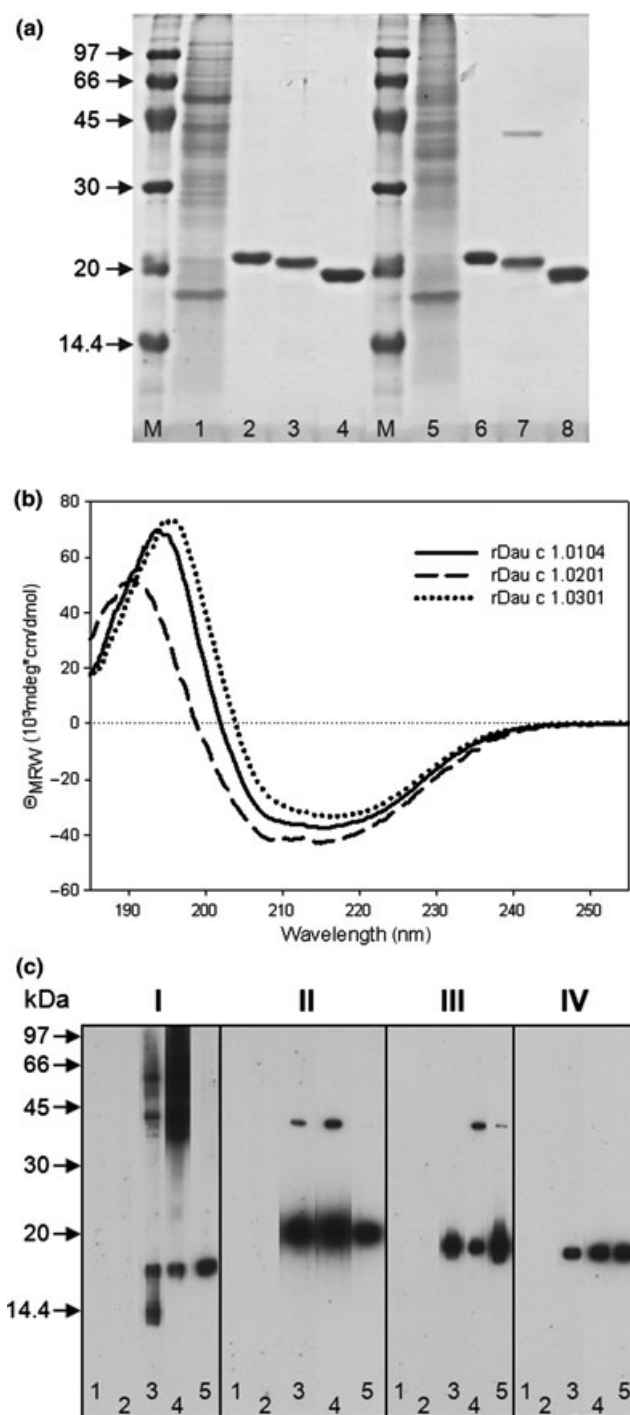


Fig. 1. (a) SDS-PAGE analysis and Coomassie staining of recombinant carrot allergens and carrot extract. M: Molecular mass marker; lanes 1 and 5: carrot extract; lanes 2 and 6: rDau c 1.0104; lanes 3 and 7: rDau c 1.0201; lanes 4 and 8: rDau c 1.0301 (Dau c PRPlike); reducing (lanes 1–4) and non-reducing conditions (lanes 5–8). (b) Circular dichroism spectra of the recombinant Dau c 1 isoforms. (c) IgE immunoblot analysis (non-reducing) of carrot extract (I) and recombinant carrot allergens (II: rDau c 1.0104; III: rDau c 1.0201; IV: rDau c 1.0301) with sera of carrot allergic patients (lanes 3–5) and non-allergic controls (lanes 1–2).

forms of the major carrot allergen, sensitization to Dau c 1 was confirmed for 73% (29/40) of the patients. For six patients (nos. 6, 12, 13, 16, 28 and 31) sensitization to neither carrot extract, recombinant Dau c 1 isoforms nor bromelain (displaying cross-reactive carbohydrates, CCD) could be demonstrated. Likewise, these patients were not sensitized to cross-reactive birch pollen allergens Bet v 1 (Table 1) or profilin Bet v 2 (data not shown). Notably, five of these six patients were skin tested with carrot extract (599a/89N Bencard) and showed a positive result. For seven of 13 patients with negative ImmunoCAP™ results using carrot extract, the diagnostic sensitivity was enhanced by recombinant Dau c 1. In contrast, in five patients (nos. 1, 2, 7, 15, and 33) without any reactivity to the rDau c 1 isoforms, IgE sensitization to carrot extract was detected. Altogether we found a limited correlation between IgE to carrot extract and the recombinant isoforms: 22 out of 27 sera, reactive to carrot extract, were detected positive to at least one of the recombinant Dau c 1 isoforms. Therefore, sensitization to carrot probably is attributed to reactivity to other carrot allergens and/or CCDs, as shown for four of five patients without sensitization to Dau c 1, but reactivity to bromelain. In summary, 65% (26/40) of the sera reacted with rDau c 1.0104, 63% (25/40) with rDau c 1.0201, 68% (27/40) with rDau c 1.0301, and 26% (10/39) with CCD, whereas a diagnostic sensitivity of 68% (27/40) was calculated for carrot extract. Mean (median) specific IgE values were 3.32 (0.84) kU_A/L for rDau c 1.0104, 2.87 (0.84) kU_A/L for rDau c 1.0201 and 3.74 (0.93) kU_A/L for rDau c 1.0301. Interestingly, all patients sensitized to rDau c 1.0301 were also sensitized to rDau c 1.0104, but not in all cases to rDau c 1.0201. In line with this, specific IgE levels to rDau c 1.0301 strongly correlated with IgE levels to rDau c 1.0104 (Fig. 2b). In contrast, correlation of specific IgE values of rDau c 1.0301 (and

rDau c 1.0104, respectively) with rDau c 1.0201 were less pronounced (Fig. 2a and c).

Dau c 1 isoforms display IgE epitope heterogeneity

Inhibition assays were performed to investigate the cross-reactivity and epitope diversity of IgE reacting with Dau c 1 isoforms. Three sera (nos. 9, 35 and 40), selected for their strong reactivity to all three rDau c 1 isoforms, were used for dose-dependent IgE competition assays. A heterogeneous inhibition pattern of Dau c 1 isoforms could be observed, depending on the serum applied (Fig. 3). Using rDau c 1.0104 on the solid phase and serum no. 40, rDau c 1.0201 showed the strongest inhibitory capacity, even stronger than rDau c 1.0104 and rDau c 1.0301 (Fig. 3a). In contrast, pre-incubation of serum no. 9 and 35 with rDau c 1.0201 had almost no effect on the IgE binding to rDau c 1.0104. In addition, rDau c 1.0301 showed a distinct inhibition capacity, of 40 up to 80%, for all three sera (Fig. 3a). Using rDau c 1.0201 on the solid phase, rDau c 1.0104 and rDau c 1.0301 displayed minor or no inhibitory capacity for sera no. 9 and 35, whereas IgE-binding to rDau c 1.0201 of serum no. 40 was completely abolished for all three isoforms (Fig. 3b). The extent of inhibition of IgE binding to rDau c 1.0301 by the two other Dau c 1 isoforms differed among the three sera used (Fig. 3c). The strongest inhibition capacity was obtained either with rDau c 1.0104 (serum no. 35), rDau c 1.0201 (serum no. 40), or with rDau c 1.0301 itself (serum no. 9). Carrot extract, used as positive control in the experimental settings, gave rise to almost complete inhibition of IgE binding to all Dau c 1 isoforms, whereas BSA as control caused no inhibition at all (Fig. 3). In summary, the cross-reactivity of IgE from different patients to the investigated allergens was found to be highly diverse and depended on the specific combination of Dau c 1

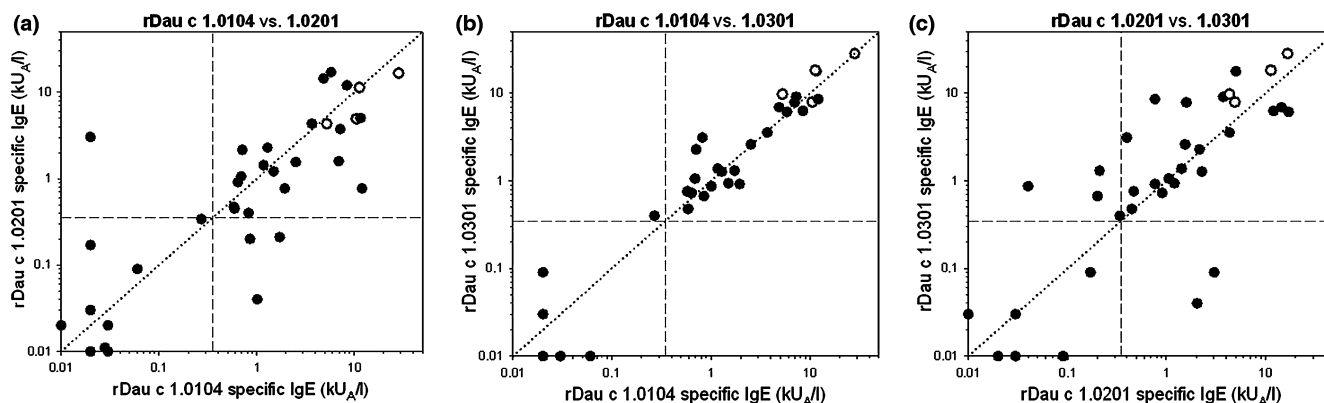


Fig. 2. Determination of IgE specific for Dau c 1 isoforms by means of experimental ImmunoCAP with sera of carrot allergic patients ($n = 40$); (a) Dau c 1.0104 vs. 1.0201; (b) Dau c 1.0104 vs. 1.0301; (c) Dau c 1.0201 vs. 1.0301. Open circles indicate sera used for IgE-inhibition and histamine release assays.

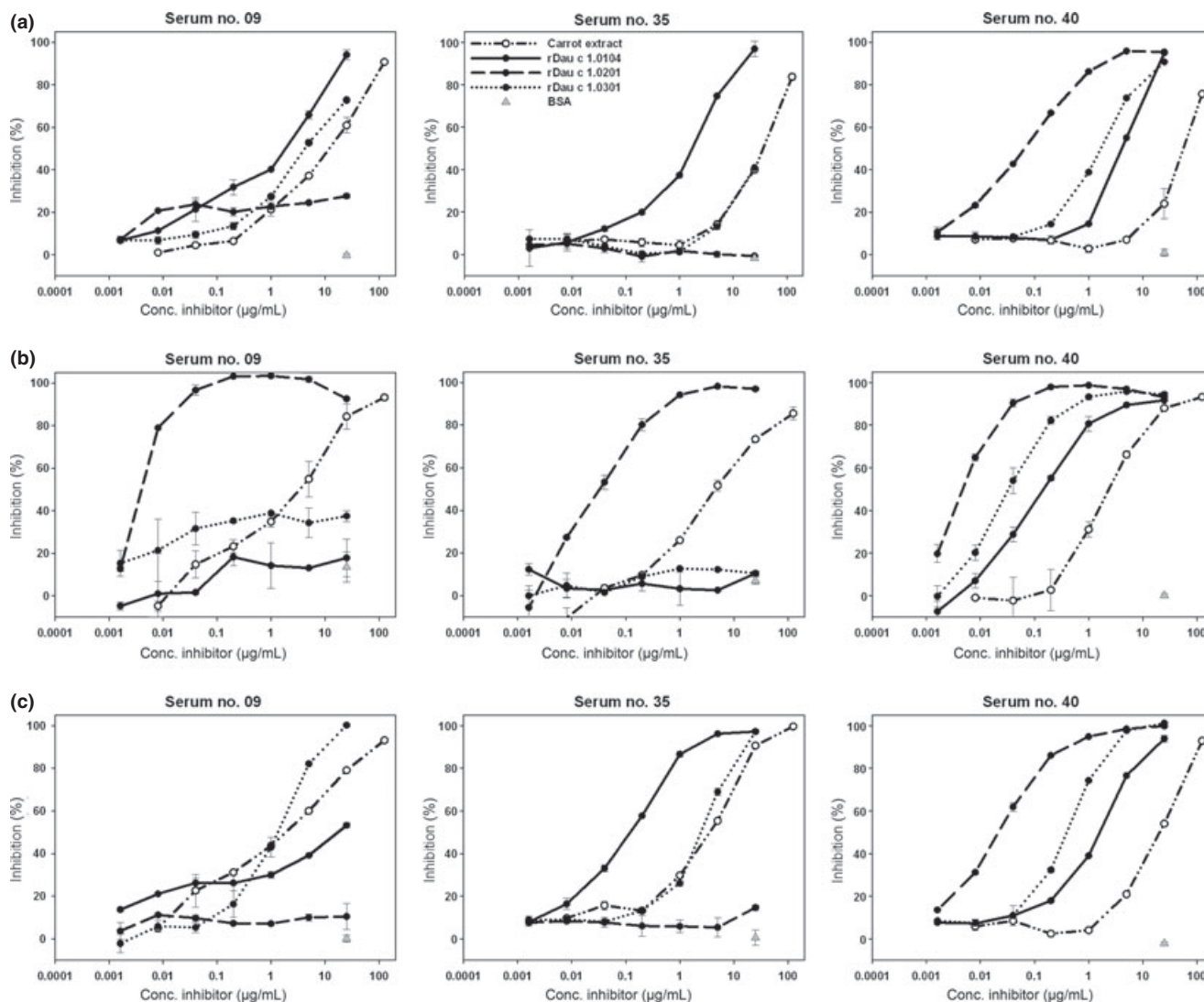


Fig. 3. Inhibition of IgE binding to rDau c 1.0104 (a), rDau c 1.0201 (b) and rDau c 1.0301 (c) by pre-incubation of sera from carrot allergic patients ($n = 3$) with Dau c 1 isoforms.

isoform and serum. Each Dau c 1 isoform seems to display both cross-reactive and unique IgE-binding epitopes.

Dau c 1.0301 is capable of triggering mediator release

Histamine release assays were performed using basophils passively sensitized using sera with reactivity to Dau c 1 isoforms (CAP classes 3 and 4). Representative results from two carrot allergic patients (nos. 17 and 35) demonstrating the allergenic potency of Dau c 1.0301 are shown in Fig. 4. All recombinant isoforms and carrot extract, applied as positive control, were able to induce histamine release. For the selected patients Dau c 1.0301 is the most potent inducer of mediator release. Although all Dau c 1 isoforms were able to cross-link bound IgE on stripped basophils and induce

mediator release, the allergenic potency of tested isoforms is different.

Dau c 1.0301 and Dau c 1.0104 share conserved surface patches

To compare conserved surface patches between the isoforms, a model of the tertiary structure of Dau c 1.0104 was compared with the deduced structures of Dau c 1.0201 and Dau c 1.0301 (Fig. 5). The comparison indicated that Dau c 1.0301 shares more surface residues with Dau c 1.0104 than with Dau c 1.0201: Dau c 1.0104 shares only 50.6% (78/154) of conserved residues with Dau c 1.0201, but 70.1% (108/154) with Dau c 1.0301. In particular the P-loop region (aa₄₁–aa₅₂), which is described as one IgE-binding epitope of the Bet v 1-like protein family [28], is conserved between

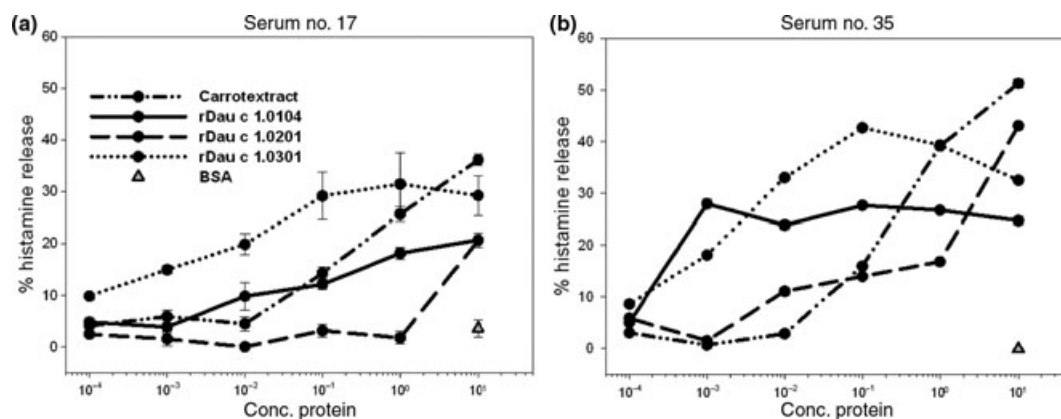


Fig. 4. *In vitro* histamine release assay with sera from two carrot allergic patients after stimulation with rDau c 1 isoforms and carrot extract. BSA was used as negative control.

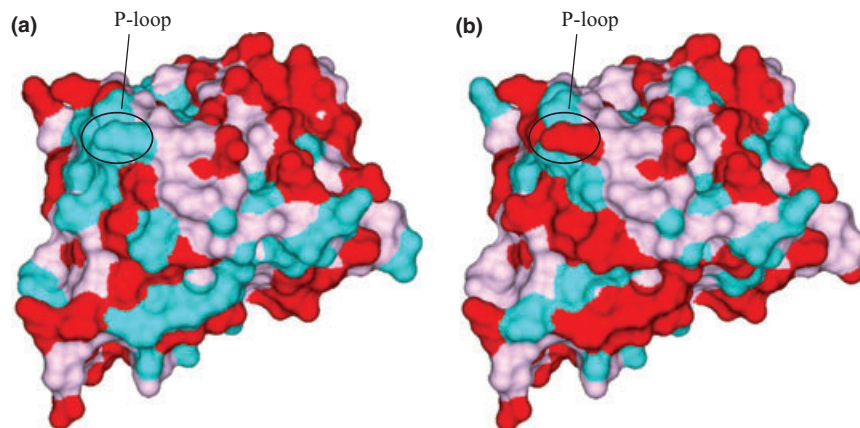


Fig. 5. Conserved surface residues of Dau c 1 isoforms: (a) between Dau c 1.0104 and Dau c 1.0201; (b) between Dau c 1.0104 and Dau c 1.0301. Conserved amino acids are indicated in red (side chains) and pink (main chain); non-conserved amino acids are indicated in cyan.

Dau c 1.0104 and Dau c 1.0301, whereas in Dau c 1.0201 amino acid position 45 is changed from lysine to glutamic acid. The comparison between Dau c 1.0201 and Dau c 1.0301 was not possible because no structure model of one of these isoforms was available to be used as template.

Dau c 1.03 mRNA transcripts are up-regulated in Dau c 1.01 and Dau c 1.02 gene-silenced transgenic carrots

To determine the expression level of Dau c 1.03 in response to biotic stress, slices of wild-type carrots, and Dau c 1.01 and Dau c 1.02 gene-silenced transgenic carrot roots (lines L39 and L10, respectively) were infected with *Alternaria radicina* (Ar). The transcripts were quantified by qPCR using Dau c 1.03 specific oligonucleotides (Fig. 6). The Dau c 1.03 gene transcript was clearly increased time-dependently in both transgenic gene-silenced lines, L10 and L39, while the wild-type carrots showed only a minor increase in the iso-

form transcripts. Moreover, as compared to the control slices in the absence of a fungal infection, a slight increase of Dau c 1.03 transcripts was observed in the *Alternaria* treated slices from transgenic lines. This result suggested a general compensation to the physiological function within DcPRPlike proteins *in planta*.

Discussion

Approximately 85% of carrot allergic patients have been reported to be sensitized to Dau c 1, the major carrot allergen, and a smaller number of patients are sensitized to carrot profilin, Dau c 4, and cross-reactive carbohydrates [29]. Until now only two isoforms of the major carrot allergen have been identified and characterized, Dau c 1.0104 and Dau c 1.0201. In addition, sequences of a group of carrot PRPlike proteins homologous to the known major carrot allergens have been deposited in the NCBI GenBank database, but were not reported as allergens so far. Therefore, this study aimed

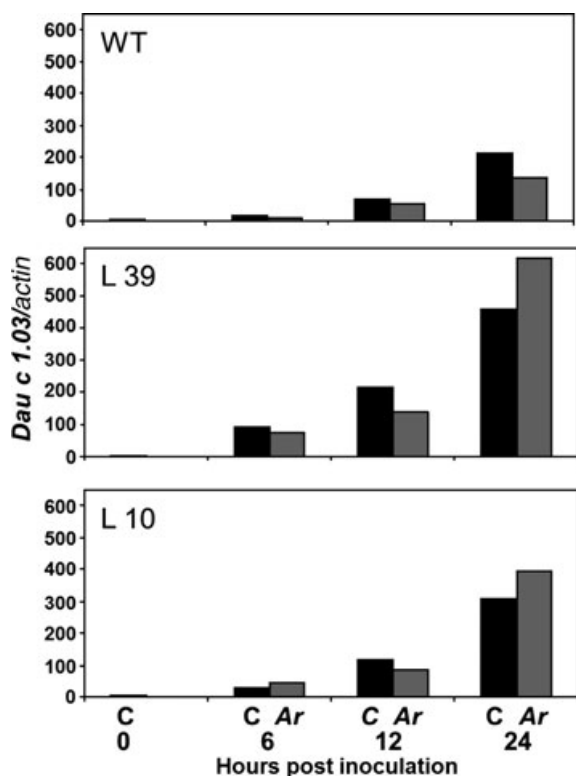


Fig. 6. Expression of Dau c 1.03 in roots of carrot plants (cv *Rodelika*) at 0, 6, 12, and 24 h after slicing and inoculation with either $10 \mu\text{L } 1 \times 10^5$ *Alternaria radicina* spores (Ar) or water [control (C)] as measured by quantitative real-time PCR. WT: Wild-type; L39: Dau c 1.01 gene-silenced transgenic line 39; L10: Dau c 1.02 gene-silenced transgenic line 10.

at investigating the allergenic properties and potential clinical relevance of a Dau c PRPlike protein in comparison to known allergenic isoforms of Dau c 1.

By RT-PCR, we demonstrated simultaneous expression of mRNA transcripts of Dau c 1.0104, Dau c 1.0201 and Dau c PRPlike proteins in carrot roots from cv *Rodelika*. cDNA analysis of Dau c PRPlike proteins identified by RT-PCR showed up to 99% aa-id with known Dau c PRPlike 1 proteins (NCBI GenBank database), but only 70% aa-id with the major carrot allergen Dau c 1.0104. Hence, Dau c PRPlike proteins were classified as Dau c 1 isoforms. To investigate allergenic properties of different Dau c 1 isoforms, one member of the identified Dau c PRPlike protein family was selected for further characterization and compared to both known Dau c 1 isoforms. All three isoforms were compared for IgE-binding capacity, allergenic potency and cross-reactivity. As determined by IgE immunoblot and ImmunoCAPTM analysis, 57% and 68%, respectively, of carrot allergic patients reacted to the recombinant Dau c PRPlike protein. Hence, the selected Dau c PRPlike protein was recognized as an allergen by the IUIS Allergen Nomenclature Subcommittee and named Dau c

1.0301 (GenBank accession no. HM064421). In comparison to immunoblotting, a superior sensitivity was achieved by ImmunoCAPTM testing, probably due to the performance of the assay under native conditions and the overall higher concentration of the antigen on the solid phase. In contrast to a previous study [9], we observed a diverse reactivity of individual patients to rDau c 1.0104 and rDau c 1.0201. Using ELISA assays, Ballmer-Weber et al. showed an overall higher prevalence of IgE-reactivity of rDau c 1.0104 in comparison to rDau c 1.0201 [9]. Different results might be due to the selection of patients, technical differences between the assays used (ImmunoCAPTM vs. ELISA) or the quality and amount of antigens applied. In the present study, IgE levels against rDau c 1.0301 showed a good correlation with rDau c 1.0104, but a clearly weaker correlation to rDau c 1.0201, which probably is due to a structure variability between isoforms. Two patients were sensitized to rDau c 1.0201 but not to the two other isoforms. *Vice versa* four patients who did not react to rDau c 1.0201 were sensitized to rDau c 1.0301 and rDau c 1.0104, respectively. Therefore, rDau c 1.0301 did not improve diagnostic sensitivity of the recombinant allergen test panel in this study population, as rDau c 1.0104 was already included. In contrast, the combination of rDau c 1.0104 or rDau c 1.0301 with rDau c 1.0201 might enhance the diagnostic sensitivity over natural carrot extract. Moreover, the results obtained by ImmunoCAPTM testing pointed towards the existence of isoform-specific IgE-epitopes.

To verify the presence of different epitopes, dose-dependent IgE-inhibition experiments were performed using three sera with confirmed reactivity to all three Dau c 1 isoforms. We found cross-reactivity of IgE between the three isoforms to be strongly dependent on the individual serum tested. Recently, Ballmer-Weber et al. reported a diverse inhibition pattern between Dau c 1.0201 and Dau c 1.0104, pointing towards epitope differences [9]. Here, depending on the serum applied, we observed a strong cross-reactivity even between rDau c 1.0201 and rDau c 1.0301 (serum no. 40), whereas other sera displayed no cross-reactive IgE (no. 9 and 35). In summary, cross-reactive properties of rDau c 1.0104 and rDau c 1.0301 are potentially higher than those between Dau c 1.0104 and Dau c 1.0201. The results corroborate structure-based predictions of IgE epitope diversity between isoforms. Analysis of conserved surface residues according to Jenkins et al. [25] showed some regions of Dau c 1.0201 and Dau c 1.0301 displaying differences in the surface residues in comparison to Dau c 1.0104. In particular the P-loop region, described as one of the IgE-binding epitopes on Bet v 1 related allergens in fruits and vegetables [27, 28, 30, 31], is less conserved between Dau c 1.0104 and Dau c 1.0201, which could explain the reduced IgE-

binding capacity of Dau c 1.0201 for some patients. Differences in the amino acids on the surface which might be involved in conformational IgE epitopes will likely have an impact on the IgE-binding properties of the Dau c 1 isoforms as determined by histamine release and IgE-inhibition assay.

Allergenic potency of the new isoform rDau c 1.0301 was confirmed by its ability to induce histamine release from passively sensitized basophils. For selected patients mediator release capacity, in respect to effector dose and maximum release, was stronger for rDau c 1.0301 in comparison to rDau c 1.0201 and rDau c 1.0104. Therefore, Dau c 1.0301 presumably contributes to the manifestation of carrot allergy. Recently a dominant T cell epitope of Bet v 1, Bet v 1_{142–156}, contributing to the cross-reactivity with homologous food allergens, e.g. Dau c 1, has been described [32]. The correlating peptide of Dau c 1.0104 (aa_{144–156}) induced proliferation of 47% of T cell lines from birch pollen allergic donors. Notably, 11 out of 13 (85%) amino acids are conserved in the corresponding peptide derived from Dau c 1.0301, indicating the possibility of a retained T cell cross-reactivity between this carrot isoform and Bet v 1.

Furthermore, the present study addressed the expression of Dau c 1.03 transcripts in wild-type (wt) and transgenic carrots (cv *Rodelika*). These transgenic carrots have been generated recently by RNAi-mediated gene silencing of Dau c 1.01 and Dau c 1.02 isoforms, respectively [11]. None of these siRNA constructs were designed to target Dau c 1.0301 and analysis of the cDNA sequences suggests that they would also not have done so by chance. Since the transgenic carrots showed only a reduced, but not abolished allergenic potency [11], we decided to assess Dau c 1.0301 mRNA abundance in carrot root tissue. Interestingly, we found a time-dependent up-regulation of Dau c 1.0301 mRNA transcripts after slicing of the carrots, both in wild-type and even more prominent in transgenic lines. Infection with *Alternaria radicina* of transgenic lines resulted in a slight, but no significant induction of Dau c 1.0301 mRNA compared to the control. We hypothesize that abiotic stress by physical injury and likely biotic stress acts to induce Dau c 1.03 and potentially other Dau c 1 PR-10 proteins, compensating for the loss of physiological function of suppressed Dau c 1 isoforms. Recently, Zagon et al. provided evidence for a high transcription

level of Dau c 1.02 mRNA in carrots grown under organic conditions [33], which likely correlates with a higher rate of pathogen infection. These findings are of particular interest with respect to allergen suppression in plants applying isoform specific small interfering RNA and the subsequent allergenicity risk assessment. In terms of simultaneous expression of several isoforms in plants the RNAi approach might have a limited value. Moreover, expression of Dau c 1.03 in carrot roots (cv *Rodelika*) was analysed by UPLC Q-TOF mass spectrometry. Despite the identification of several different Dau c 1 isoforms, Dau c 1.03 has not been detected (not shown). Therefore, we conclude there is a minor clinical relevance of Dau c 1.03 in wild-type carrots. Nevertheless the possibility of up-regulation of Dau c 1.03 upon biotic and abiotic stress, in particular in Dau c 1.01- and Dau c 1.02-silenced carrots needs to be investigated.

In summary, a Dau c PRlike protein has been identified as new allergenic isoform in carrot roots and was denominated Dau c 1.0301. Carrot allergic patients showed an individual IgE-reactivity pattern to Dau c 1 isoforms, indicating epitope diversity between these carrot allergens. The application of rDau c 1.0201 and rDau c 1.0301 or Dau c 1.0104 enhances the diagnostic sensitivity in comparison to carrot extract. Moreover, Dau c 1.03 transcripts are likely up-regulated under stress conditions in carrot roots. Therefore, knowledge about isoform expression and distribution in plants is of particular interest for allergen suppression in plants and for allergy diagnosis.

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Conflict of Interest: Stefan Vieths has received speaker's honoraria from Phadia, Germany and Fresenius Akademie, Germany. He served as a paid consultant as expert reviewer for the FARRP Allergen Online database. SV has also received research funding from Monsanto Company and Pioneer Hi-Bred International, Inc. Jonas Lidhom and Åsa Marknell DeWitt are employees of Phadia AB. All other authors have declared no conflict of interest.

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