

Dau c 1.01 and Dau c 1.02-silenced transgenic carrot plants show reduced allergenicity to patients with carrot allergy

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Abstract Pathogenesis-related protein-10 (PR10) is a ubiquitous small plant protein induced by microbial pathogens and abiotic stress that adversely contributes to the allergenic potency of many fruits and vegetables, including carrot. In this plant, two highly similar genes encoding PR10 isoforms have been isolated and designated as allergen Dau c 1.01 and Dau c 1.02. The aim of the study was to generate PR10-reduced hypoallergenic carrots by silencing

either one of these genes in transgenic carrots by means of RNA interference (RNAi). The efficiency of gene silencing by stably expressed hairpin RNA (hnRNA) was documented by means of quantitative RT-PCR (qPCR) and immunoblotting. Quantification of the residual protein revealed that PR10 accumulation was strongly decreased compared with untransformed controls. Treatment of carrot plants with the PR protein-inducing chemical salicylic acid resulted in an increase of PR10 isoforms only in wild-type but not in Dau c 1-silenced mutants. The decrease of the allergenic potential in Dau c 1-silenced plants was sufficient to cause a reduced allergenic reactivity in patients with carrot allergy, as determined with skin prick tests (SPT). However, simultaneous silencing of multiple allergens will be required to design hypoallergenic carrots for the market. Our findings demonstrate the feasibility of creating low-allergenic food by using RNAi. This constitutes a reasonable approach to allergen avoidance.

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Hypoallergenic food

Abbreviations used

Qpcr	Quantitative RT-PCR
PR	Pathogenesis-related
RNAi	RNA interference
SPT	Skin prick test
WT	Wild-type

Introduction

PR10 proteins comprise 1 of the currently 17 groups of pathogenesis-related plant proteins (Christensen et al. 2002) that are part of the defence system protecting plants from pathogen challenge and abiotic stresses (Van Loon and Pieterse 2006). Unlike most other PR proteins with extracellular destination, PR10 are typically small (16–19 kDa), acidic and intracellularly located proteins (Liu and Ekramodoullah 2006). In connection with clinical studies, PR10 proteins have been described as panallergens in several food sources, in particular fruits, vegetables, and tree nuts as well as in pollen (Vieths et al. 2002). PR10 proteins show a high degree of amino acid sequence identity with the birch pollen allergens of the Bet v 1 family and are the most important elicitors of pollen-related food allergy. Approximately 90% of patients allergic to birch pollen have IgE antibodies against Bet v 1 (Jahn-Schmid et al. 2005).

Carrot (*Daucus carota*) allergy is also a common phenomenon in patients with birch pollen allergy. The carrot allergen Dau c 1 is a homologue of Bet v 1 (Hoffmann-Sommergruber et al. 1999). Recently, a new isoform of Dau c 1, denominated Dau c 1.02, was identified (Ballmer-Weber et al. 2005). This isoform showed only 50% amino acid sequence identity with the known isoform Dau c 1 (then denominated 1.01), and limited cross-reactivity was observed between both isoforms, and also with Bet v 1 in some patients. Because limited cross-reactivity to Bet v 1 was also observed at the T cell level (Bohle et al. 2005), it was concluded that Dau c 1.02 may represent a partially pollen-independent stimulus to the immune system. In contrast to Bet v 1-related allergy to apple, food allergy caused by Dau c 1 tends to be more severe, with approximately 50% of patients experiencing systemic reactions (Ballmer-Weber et al. 2001). Dau c 1 represents the major allergen in carrot, and up to 60% of carrot allergic patients are mono-sensitized to this protein.

One goal of the present work was generating carrot plants with attenuated allergenicity due to reduced expression of the Dau c 1 protein. The presence of more than one isoform of this allergenic protein is only one reason that makes conventional breeding of a hypoallergenic carrot plant a complex and extremely time-consuming approach. For instance, because of its possible function in plant defence and development, it

is reasonable to suppress activity of potentially allergenic Dau c 1 proteins in a tissue-specific manner only in edible plant organs. In the long run, such a strategy could avoid weakening plant performance in its environment as much as possible. By following conventional breeding methods, tissue-specific silencing of gene families cannot easily be achieved. However, it is possible by exploiting tissue-specific plant promoters in gene silencing strategies. Different gene-silencing procedures have been applied in soybean, rice, apple, and tomato to decrease the accumulation of allergenic proteins thereby creating low-allergenic food sources (Nakamura and Matsuda 1996; Tada 1996; Herman 2003; Gilissen et al. 2005; Le et al. 2006). Gene silencing by means of RNA interference (RNAi) technology offers the possibility to inhibit expression of entire gene families. This aim can be achieved by selecting nucleotide regions of high similarity often found within coding regions. In contrast, selecting less conserved 5'- or 3'-untranslated regions allows inhibition of individual members of gene families. Although systemic silencing has been shown for non-plant target genes (i.e., viruses), silencing of endogenous genes seems to be cell autonomous (Ossowski et al. 2008). Thus, by selecting appropriate promoter sequences, fruit-, seed-, or pollen-specific silencing of target genes can be envisaged.

Here, we report on the successful respective inhibition of Dau c 1.01 and 1.02 expression in transgenic carrot plants by constitutive expression of Dau c 1.01- and Dau c 1.02-specific hairpin RNAs (hnRNA). On the basis of a quantitative PCR analysis (qPCR) and immunoblotting, accumulation of Dau c 1.01 and 1.02 was confirmed to be strongly reduced compared with untransformed carrots. When tested with carrot extracts from Dau c 1.01- and 1.02-silenced plants, patients with carrot allergy had strongly reduced SPT reactivity, indicating a reduced allergenic reactivity in vivo caused by suppression of Dau c 1 expression.

Results

Dau c 1 isoforms are up-regulated upon biotic stress

Since the carrot allergens of the group Dau c 1 (synonymic DcPR10) are expected to function as

pathogenesis-related protein, we were interested to investigate whether both isoforms Dau c 1.01 and Dau c 1.02 were induced by a microbial pathogen under natural conditions. To this end, we harvested 4-month-old carrot plants grown under field conditions and challenged carrot slices with conidia of the fungal pathogen *Alternaria radicina*. We found that transcripts of both isoforms strongly accumulated from 6 h (for Dau c 1.02) and 24 h (for Dau c 1.01) onwards, indicating that both allergens are strongly responsive to biotic stress (Fig. S1).

Dau c 1-reduced carrots by means of RNAi

An RNAi strategy was followed to generate carrots with reduced Dau c 1 expression. The two isoforms, Dau c 1.01 and Dau c 1.02, share approximately 50% amino acid identity and 66% nucleotide identity (Ballmer-Weber et al. 2005). Empirically, the degree of similarity was not sufficient to allow silencing of both isoforms by one RNAi construct. Thus, a 253-bp cDNA fragment of Dau c 1.01 [position nucleotides (nt) 1–253] and a 267-bp cDNA fragment of Dau c 1.02 (position nt 56–323) were inserted separately in sense and antisense orientation into the plant transformation vector pK7GWIWG2(II) (Fig. S2). The RNAi constructs were transformed separately into carrot cells by *Agrobacterium tumefaciens*-mediated gene transfer, and transgenic plants were generated independently on kanamycin-containing medium via somatic embryogenesis. To validate the silencing of the two isoforms, extracts from 4-month-old transgenic plants were screened for Dau c 1.01 and Dau c 1.02 mRNA and protein expression. Total RNA from carrot roots and leaves were isolated, translated into cDNA and subjected to qPCR analysis. Table 1 shows exemplarily the relative expression values in

carrot leaves and roots for wild-type (WT) carrot Rodelika, Dau c 1.01-silenced lines 35 and 39, and Dau c 1.02-silenced line 10. The relative expression levels of both isoforms were generally much higher in roots than in leaves. Moreover, expression of Dau c 1.02 in leaves and roots was always four to sixfold higher compared to Dau c 1.01. In the transgenic lines, expression of the respective Dau c 1 isoform was strongly suppressed.

Dau c 1 proteins are strongly reduced in transgenic carrot roots

Dau c 1-isoform-specific antibodies were applied for comparison of allergen accumulation in WT (mixture of 8 mock samples) as well as Dau c 1.01_RNAi and Dau c 1.02_RNAi carrot roots (Fig. 1). In immunoblots, the mouse monoclonal antibody M3 recognized the natural Dau c 1.01 isoform in the WT extract, but not in the extracts of Dau c 1.01_RNAi transgenic lines 8, 25, 35, and 39 (Fig. 1a). The antibody M9 detected Dau c 1.02 in the WT extract, but not in the Dau c 1.02_RNAi lines 10, 28, and 33 (Fig. 1b). Both monoclonal antibodies were reactive with their respective recombinant Dau c 1 isoform and are not cross-reactive (data not shown). The slightly higher molecular masses of recombinant Dau c 1.01 and Dau c 1.02 in comparison to natural counterparts are due to residual His-tags of the proteins.

Dau c 1.01 and Dau c 1.02 remain silenced under salicylic acid treatment

In order to validate a substantial suppression of Dau c 1 isoforms in the silenced carrot lines even when challenged by chemical stress, we tested their

Table 1 Dau c 1.01- and Dau c 1.02-silencing rates in leaves and roots

	Relative expression (qPCR)			
	Leaf		Root	
	Dau c 1.01	Dau c 1.02	Dau c 1.01	Dau c 1.02
Wild-type	0.5120	14.8288	10.5484	45.5756
L35 (Dau c 1.01_RNAi)	0.1117 (4.5-fold)	7.8989 (1.8-fold)	1.3213 (7.9-fold)	40.1683 (1.1-fold)
L39 (Dau c 1.01_RNAi)	0.0071 (72-fold)	n.d.	0.8235 (12.8-fold)	28.7435 (1.6-fold)
L10 (Dau c 1.02_RNAi)	0.1057 (4.8-fold)	0.4916 (30.2-fold)	0.7817 (13.5-fold)	0.3214 (141.8-fold)

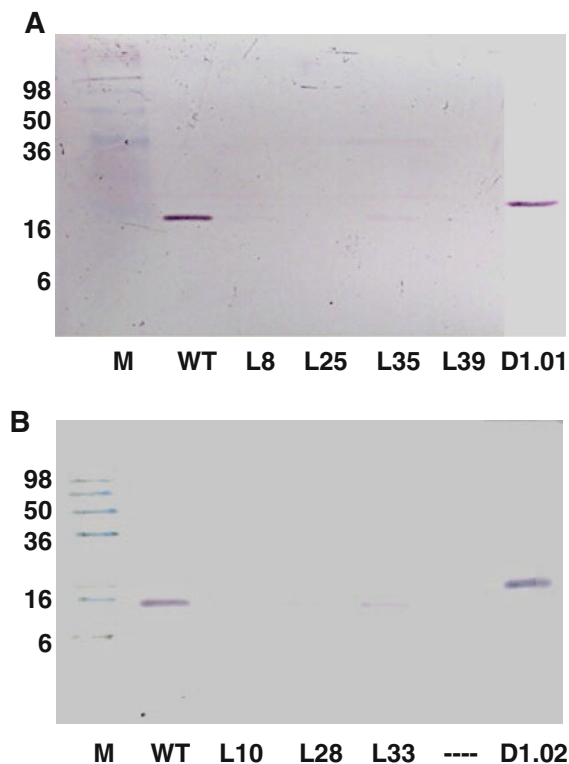


Fig. 1 Immunodetection of Dau c 1 in protein extracts from carrot root (cv Rodelika) of wild-type (WT, mix of 8 mock samples) and Dau c 1.01_RNAi transgenic lines 8, 25, 35, and 39 (0.8 µg/lane) by using monoclonal antibody M3 directed against Dau c 1.0104 (**a**) and Dau c 1.02_RNAi transgenic lines 10, 28, and 33 (0.1 µg/lane) by monoclonal antibody M9 directed against Dau c 1.0201 (**b**). Recombinant rDau c 1.0104 (D1.01) and rDau c 1.0201 (D1.02), 5 ng each, were used as positive controls. M SeeBlue®Plus2 marker (*numbers on the left side* represent molecular weights of marker proteins)

responsiveness to salicylic acid (SA). This chemical is known to be a strong inducer of systemic acquired resistance and PR gene expression in a wide range of plant species (Kogel and Langen 2005). Consistently, spraying leaves of WT carrots with an aqueous solution of 1 mM SA induced the PR10 isoforms, Dau c 1.01 and Dau c 1.02, by approximately 24 h after treatment (Fig. 2). In contrast, accumulation of Dau c 1.01 remained reduced upon spraying with SA in Dau c 1.01_RNAi lines (exemplarily shown for L35), while Dau c 1.02 remained reduced in Dau c 1.02_RNAi lines (exemplarily shown for L10). This data shows that RNAi-mediated silencing of the isoforms of the major carrot allergen Dau c 1 is substantially suppressed even under PR gene-inducing conditions.

IgE-reactivity is strongly reduced in Dau c 1_RNAi lines

Ten patients with a history of clinically relevant carrot allergy were included to test the allergenic reactivity to Dau c 1.01- and Dau c 1.02-reduced carrots. Specific IgE against carrot was confirmed by using ImmunoCAP (Phadia). Patient 1 was mono-sensitized to Dau c 1.02, whereas all other patients (2–10) had both Dau c 1.01- and Dau c 1.02-specific IgE (Table S1). The IgE-reactivity pattern was reflected by the IgE immunoblot results on separated extracts of WT and two selected transgenic lines. Sera from all carrot allergic patients except patient 1 detected the major carrot allergen Dau c 1 with an apparent molecular mass 16 kDa in carrot extracts from WT (Fig. 3a) and from the Dau c 1.02_RNAi transgenic line 33 (Fig. 3c). In contrast, only sera from patients 2 and 9, showing the strongest binding to natural Dau c 1, recognize residual Dau c 1 in Dau c 1.01_RNAi transgenic line 39 (Fig. 3b, lanes 2 and 9). Four out of 10 sera tested showed IgE-binding to proteins with an apparent molecular mass between 36 and 98 kDa, likely not yet identified carrot allergens, or glycoproteins expressing IgE cross-reactive carbohydrates. As negative controls, a serum from one non-allergic patient and a buffer control were included.

Transgenic carrot roots show reduced allergenic potential in skin prick test

In the SPTs the wheal-and-flare reactions to Dau c 1.01- and to Dau c 1.02-reduced carrots differed between individual patients with Dau c 1 allergy and were dependent on the individual profile of sensitization (mono-sensitized to one allergen or sensitized to several carrot allergens) and on the *in vivo* recognition of these additional carrot proteins in different carrot lines.

In a patient with mono-sensitization to Dau c 1.02 (Fig. 4 and Supplementary Table S1 patient 1), an average reduction of the mean wheal reaction (MWR) of 50% occurred with Dau c 1.02-reduced (L 10) carrots compared with WT. In poly-sensitized patients with sensitivity to both PR 10 isoforms (Supplementary Table S1, patients 6 and 8) or with additional sensitization to further carrot allergens, this effect was less pronounced [average reduction of MWR to Dau c 1.01-reduced L 35: 21% (patient 6),

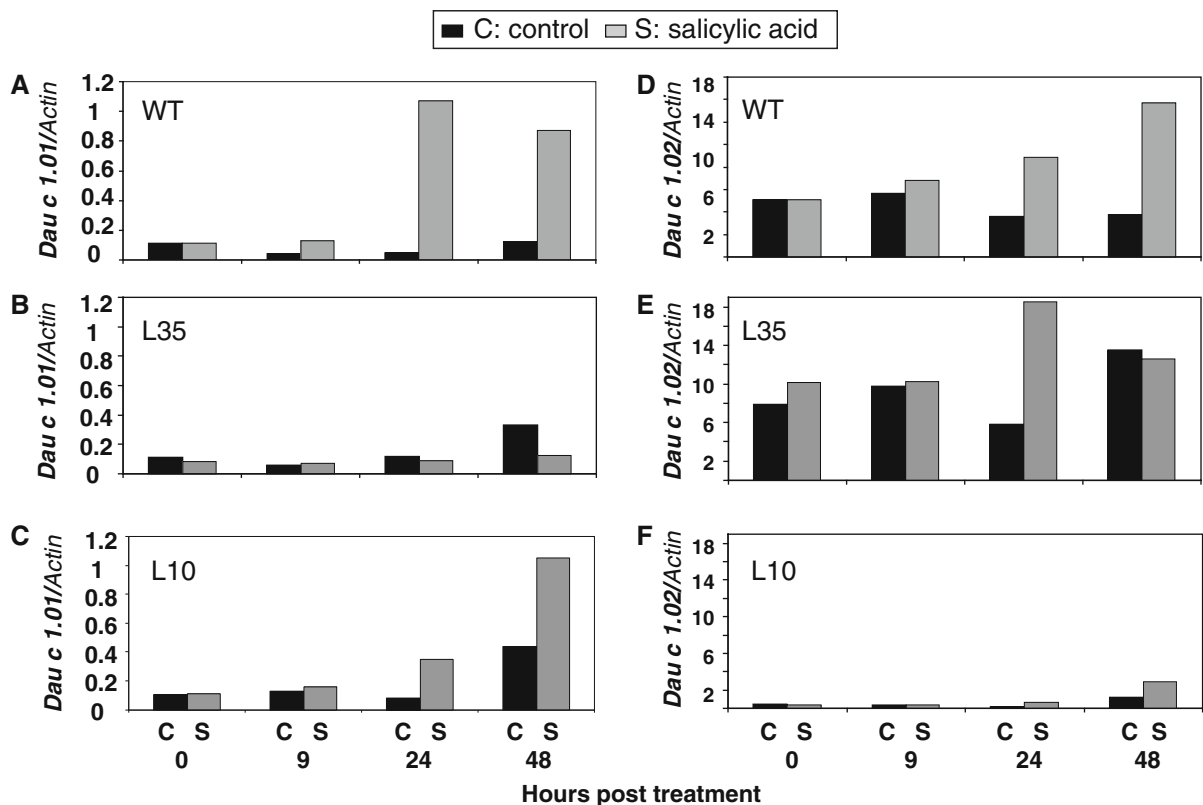


Fig. 2 Expression of the isoforms Dau c 1.01 (a–c) and Dau c 1.02 (d–f) in leaves of carrot plants at 0, 9, 24, and 48 h after treatment (hat) with either 1 mM salicylic acid (S) or H₂O [control (C)] as measured by quantitative real-time PCR. WT Wild-type Rodelika, line 35 (L35) (Dau c 1.01_RNAi), and line 10 (L10) (Dau c 1.02_RNAi). Statistically significant

differences were detected by covariance analysis of treatment effect and treatment $\times$ time interactions in the relative expression of Dau c 1.01 in leaves of line 10 (c) and in the relative expression of Dau c 1.02 in WT leaves (d). Values are given relative the actin RNA

36% (patient 8), and to Dau c 1.02-reduced L 10: 39% (patient 8) compared to WT], or not detectable (e.g., to Dau c 1.02-reduced L 10 in patient 6, Fig. 4).

Discussion

To date, the sole practised strategy for managing food hypersensitivity involves strict avoidance of the trigger. However, modern breeding-based approaches might be an alternative in future therapies. Recent advances in plant biotechnology have enabled the complete silencing of specific genes, including a gene from apple that encodes an allergenic protein of the PR 10 family (Gilissen et al. 2005). In the present work, evaluation of allergen suppression by using isoform-specific antibodies clearly demonstrates the efficiency of gene silencing by the RNAi method.

Accumulation of the main carrot allergen Dau c 1 isoforms was strongly reduced in transgenic carrots in comparison to the WT extract (Fig. 1). Allergen suppression was confirmed for the Dau c 1.01_RNAi lines 8, 25, 35, and 39 and for Dau c 1.02_RNAi line 10, 28, and 33. Results are in accordance to the results of qPCR measurements (Table 1).

Using patient sera, a residual IgE-reactivity of Dau c 1.01 could be detected in Dau c 1.01_RNAi transgenic carrots with 2 out of 10 sera tested. The results showed a reduction of IgE-reactivity which is in line with a reduced Dau c 1 content in this transgenic line. Although Dau c 1.02 suppression was confirmed by the reduced IgG-reactivity of M9, the IgE-sensitization pattern of transgenic Dau c 1.02_RNAi carrot roots was similar to WT. Results can be explained by the difference in the expression of both isoforms. Low expression of Dau c 1.02 in carrot roots explains

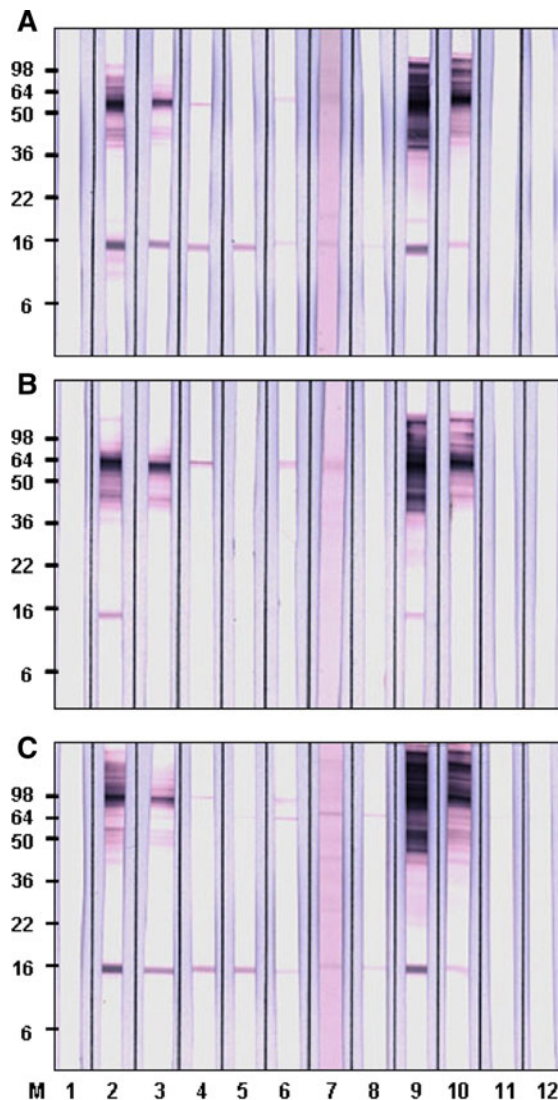


Fig. 3 IgE reactivity of sera from carrot allergic patients (lanes 1–10) with extracts from wild-type carrots (a), Dau c 1.01_RNAi (b), and Dau c 1.02_RNAi transgenic lines (c) separated by SDS-PAGE (20 µg/cm). Lane 11 Serum from non allergic control, lane 12 buffer control

non-reactivity of patient 1, who is mono-sensitized to Dau c 1.02, to any of the extracts. Since all other carrot patients also possess specific IgE to Dau c 1.01 that is not targeted by Dau c 1.02_RNAi silencing nor efficiently reduced by co-suppression, sera show the same reactivity to both the WT and the Dau c 1.02_RNAi extract. In summary, the Dau c 1_RNAi strategy has been proven to generate transgenic lines with strongly reduced Dau c 1 isoforms and confirms the efficacy of the gene silencing approach. As shown in

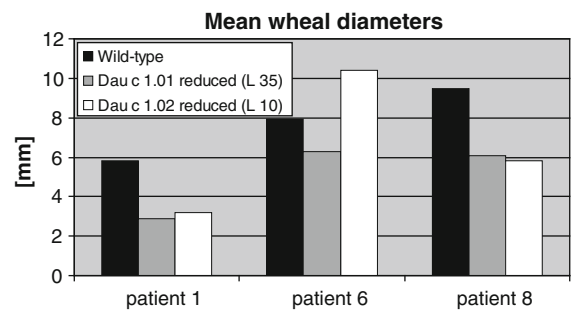


Fig. 4 Mean wheal diameters of skin prick tests (prick-to-prick) with roots from wild-type carrots (WT), Dau c 1.01_RNAi, and Dau c 1.02_RNAi transgenic lines in representative carrot allergic patients (patient 1, 6 and 8 from Supplementary Table S1)

Fig. 1, both isoforms were specifically suppressed. Potential effects on co-silencing of Dau c 1 isoforms or homologous proteins, and eventually the individual reactivity of patients has to be further evaluated.

RNAi-mediated gene silencing is gene specific. In case multiple genes or isogenes contribute to clinical reactivity, application of single siRNA might often be sufficient to generate hypoallergenic foods providing a substantial benefit for the patients. Alternatively, multi-target RNAi vectors might be considered to reach the desired aim. The RNAi method for posttranscriptional gene silencing is highly efficient especially when a gene construct is used that comprises an inverted repeat of a fragment of the targeted gene sequence separated by an intron. Such a construct results in the formation of a so-called intron-spliced hairpin (hn) RNA. This method operates by sequence-specific RNA degradation. The sequence specificity of the method enabled us to knock-down the specific isoforms Dau c 1.01 and Dau c 1.02 of the major carrot allergen Dau c 1. Since co-suppression was particularly low, this isoform-specific approach allowed us to study the contribution of each Dau c 1 isoform in allergenic reactions.

Further analysis is required to gain a full picture of the benefits of gene silencing via hnRNA in producing hypoallergenic carrots for patients with food allergies. Presently, the approach is limited by several factors. For instance, there is a possibility of increased allergenicity due to the overabundance of compensatory proteins including as yet unidentified isoforms which are not targeted by the silencing approach. Also, a concern is the possibility of reversion of the silencing effect. Reappearance of the suppressed trait could be dangerous even if reversion involves only a portion of the crop

population, because exposure to even small amounts of allergen can have serious consequences for those with severe allergy (Hompeš et al. 2010). Additionally, apart from the major allergen Dau c 1, carrot plants have minor allergens such as profilin (Dau c 4) and glycoproteins with a molecular weight of approximately 40–60 kDa that have not been characterized in detail (Ballmer-Weber et al. 2001, 2005). Further candidates representing carrot allergens are isoflavone reductase-like protein (Karamloo et al. 2001) and pectin esterase that may represent one of the unidentified glycosylated allergens in carrot. It is still an issue as to whether these minor allergens become more important in plants with a silenced major allergen Dau c 1.

From the plant pathology point of view, it is a central issue that crops with silenced pathogenesis-related proteins might be more susceptible to pest and diseases and in the long run thus cause more pesticide use. Recent work shows that individual PR10 isoforms add to the antimicrobial defence in various plant species (Chen et al. 2010; Xie et al. 2010). However, initial screens of Dau c 1-silenced carrots unexpectedly suggest that these transgenic lines are as resistant as WT plants to the fungal pathogen *Alternaria radicina* (unpublished data). One explanation for this observation is that compensatory PR proteins may strengthen the silenced lines against microbial attack thereby compensating for the suppression of PR10. This notion of a compensatory defence mechanism is also supported by Colditz et al. (2007) who showed that silencing of *MtPr10-1* from the plant *Medicago truncatula* led to induction of a set of PR proteins after infection with the fungal pathogen *Aphanomyces euteiches*. Also, individual members of the PR10 gene family including Dau c 1.01 and Dau c 1.02 may play more specific roles during stress adaptation to various pathogens. Yet, it is not clear whether the distinct isoforms may have overlapping functions and are able to partially substitute for each other during plant defence.

Experimental procedures

Plant material and growing condition

Transgenic Dau c 1.01 and Dau c 1.02-reduced and untransformed carrot plants (*Daucus carota*, L. ssp. *sativus* cultivar Rodelika) were grown via somatic embryogenesis from calli to plantlets (Imani et al.

2002) under 16 h light/8 h dark intervals (light intensity approx. $47 \mu\text{mol m}^{-2} \text{s}^{-1}$) at 24°C on B5-medium (Gamborg et al. 1968). In vitro grown plants were transferred onto a 2:1 substrate mixture of Seramis®-expanded clay (Mars, Verden, Germany) and Oil Dri (Damolin, Fur, Denmark), and acclimated and cultivated in a walk-in chamber under 16 h light/8 h dark intervals, a light intensity of $160 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 18°C. Four-week-old plants were transferred to substrate mixture of soil and sand [5:1 (v:v)] and cultivated in a plant grow chamber (Vötsch, Balingen, Germany) under the same light conditions. Two weeks later, the environmentally adapted plants were transferred to the greenhouse with 16 h supplemental light with an intensity of $160 \mu\text{mol m}^{-2} \text{s}^{-1}$. The temperature regimen followed a day/night cycle of 20°C/16°C.

Pathogen tests with carrot root slices; SA treatment of carrot plants

In order to prepare nearly homogenous materials for experiments the middle parts of approximately 4-month-old fresh roots of WT and transgenic carrots were cut in 0.5-cm-thick slices and placed on moist filter paper in a petridish. The slices were inoculated with 10 μl aqueous suspension containing 10^5 spores from *Alternaria radicina* isolate KGHN3 (JKI Que-dlingburg), and incubated at RT in relative airtight plastic boxes in the dark. Infections were recorded over 7 days. Transcripts of Dau c 1.01 and Dau c 1.02 were determined by qPCR at 0, 6, 12, 24, and 72 h after inoculation.

An aqueous solution containing 1 mM SA was sprayed onto leaves of approximately 5-month-old carrots. For the SA kinetics, 5 plants per treatment and line were pooled.

Construction of Dau c-RNAi vectors, generation of transgenic carrots

RNA was extracted from 6-week-old carrot plants by plant RNeasy kit (Qiagen, Hilden, Germany) and the cDNA was synthesized by Super Script II RT (Invitrogen) according to the manufacturer's protocols. For the Dau c 1.01-RNAi construct, a 253-nt cDNA fragment of Dau c 1.0104 (accession No. Z81362; nt 1–253) was amplified by means of PCR using the primers Dau c -1.01fwd (5'-ATGGGTGCCAGAGCCATTC-3') and Dau c -1.01rev (5'-CTTGACATA

CGACTCCACCG-3'). For the Dau c 1.02-RNAi construct, a 267 nt cDNA fragment of Dau c 1.0201 (accession No. AF456481; nt 56–323) was amplified using the primers Dau c -1.02fwd (5'-GTATCAGG GATTTCCTTGATATG-3') and Dau c -1.02rev (5'-CAATTCGTTGTTGTGCCAAC-3'). After an initial activation step at 95°C for 3 min, 34 cycles (95°C for 20 s, 60°C for Dau c 1.01, 56°C for Dau c 1.02, 60 s, 72°C for 30 s, and 68°C for 10 min) were performed. The PCR product was introduced into pENTR/D-TOPO vector (Invitrogen) and subsequently inserted into plant transformation RNAi vector pK7GWIWG2(II) (Karimi et al. 2002) by means of a lambda reconstruction (LR) recombination reaction (Invitrogen) in sense and antisense orientation under control of the constitutive cauliflower mosaic virus (CaMV35s) promoter (Fig. S2).

The final constructs were transferred to *Agrobacterium tumefaciens* AGL1, carrying the hypervirulence attenuated tumor-inducing helper plasmid pTiBo542 (Lazo et al. 1991). Carrot cells originally derived from cultured petiole explants were transformed and regenerated as described (Imani et al. 2002). The transgenes were confirmed by PCR (data not shown).

Real-Time PCR and transcript analysis

RNA was isolated from homogenized carrot roots and leaves by TRIZOL[®] Reagent (Invitrogen) as described by the manufacturer. Aliquots (1 µg) of total RNA were used for cDNA synthesis with a QantiTect[®] RT (Qiagen). The amounts of Dau c 1.01 and Dau c 1.02 were quantified by qPCR. Aliquots of 7.5 ng cDNA were used as template. A 123-bp cDNA fragment of Dau c 1.01 (accession No. Z81362; nt 234–357) was amplified by means of qPCR using the primers Dau c 1.01_fwd (5'-CTTGACATACGACTCCACCG-3') and Dau c 1.01_rev (5'-TACCAAGACCACG GCCATA-3'). A 147-bp cDNA fragment of Dau c 1.02 (accession No. AF456481; nt 17–164) was amplified by using the primers Dau c 1.02_fwd (5'-TGAGGTTG AGGCTCCTTCC-3') and Dau c 1.02_rev (5'-GTG GTGTTGGAACCGTCAG-3'). A 151-bp cDNA fragment of constitutively expressed ubiquitin (accession No. U44983; nt 152–303), or a 169-bp cDNA fragment of constitutively expressed actin was amplified by qPCR using the primers Ubi_fwd (5'-AAGCCCAA GAAGATCAAGCA-3') and Ubi_rev (5'-TTCA

TGGCCAATCATTTTGA-3'), and for actin Actin_fwd (5'-CCACTGAACCCAAAAGCAA-3') and Actin_rev (5'-CCGTGTGGCTAACACCATC-3') served as internal standard.

Preparation of recombinant Dau c 1 and protein extracts from carrot roots

Two isoforms of the major carrot allergen Dau c 1, i.e. Dau c 1.0104 (called Dau c 1.01 herein; GenBank accession No. Z81362) and Dau c 1.0201 (called Dau c 1.02 herein; accession No. AF456481) were over-expressed in *Escherichia coli* and purified as described (Ballmer-Weber et al. 2005). The recombinant allergens were highly pure and correctly folded showing different contents of secondary elements in circular dichroism spectroscopy (data not shown).

Approximately 2 g of frozen carrot roots were cut into small pieces and transferred into 4 ml PBS buffer (pH 7.1, 150 mmol/L) on ice. Samples were homogenized using an ultraturrax device (ULTRA-TURRAX T25, IKA-Labortechnik, Staufen, Germany). After protein extraction by overhead rotation for 1 h at 4°C, the samples were centrifuged for 30 min at 12,500g and 4°C. The supernatants were filtered through 5- and 1.2-µm cellulose acetate filters (Minisart; Sartorius Stedim Biotech, Göttingen, Germany) and stored either at –20°C or after shock freezing in N₂ at –80°C for long-time storage.

The protein content of the samples was determined according to the method of Bradford (Bradford 1976) with the Roti-Nanoquant protein assay solution (Roth, Karlsruhe, Germany).

Selection and characterization of patients with carrot allergy

Ten patients with a history of clinically relevant carrot allergy were recruited to test the allergenic reactivity to Dau c 1.01_RNAi and Dau c 1.02_RNAi transgenic carrot roots. The clinical and serologic characterization of patients with carrot allergy is given in Supplementary Table S1. Determination of allergen specific IgE concentration in patient sera was performed either by experimental or commercial ImmunoCAP[™] (Phadia, Uppsala, Sweden) tests. Experimental ImmunoCAP[™] carrying recombinant Dau c 1 and Dau c 4 (profilin) carrot allergens were prepared according to Marknell

DeWitt et al (2002) and assays were performed using an ImmunoCAP 250 instrument (Phadia) according to the manufacturer's instructions. Specific IgE values are expressed as kU_A/L, CAP class 0 (negative): <0.35 kU_A/L, class 1: 0.35 to ≤0.7 kU_A/L, class 2: >0.7 to ≤3.5 kU_A/L, class 3: >3.5 to ≤17.5 kU_A/L, class 4: >17.5 to ≤50 kU_A/L, class 5: >50 to ≤100 kU_A/L, class 6: >100 kU_A/L.

SDS-PAGE and immunoblotting

For IgG immunoblotting, protein extracts from WT, transgenic lines Dau c 1.01_RNAi (0.8 µg/lane) and Dau c 1.02_RNAi (0.1 µg/lane), and purified rDau c 1 isoforms (5 ng/slot) were subjected to SDS-PAGE [T: 15% (v/v)] under non-reducing conditions, and thereafter transferred onto nitrocellulose membranes (NC, 0.45 µm) by tank blotting (Macherey-Nagel, Düren, Germany). The membranes were blocked twice for 15 min with blocking buffer (TBS pH 7.4, 0.3% Tween20) and incubated for 2 h with either the mouse monoclonal antibody M3 (Dau c 1.01 isoform-specific) diluted 1:50 in incubation buffer (TBS, 0.05% Tween20), or with the mouse monoclonal antibody M9 (Dau c 1.02 isoform-specific) diluted 1:500. Specificity of mouse monoclonal antibodies M3 and M9 was confirmed by screening of hybridoma cells (data not shown). Antibody binding was visualized by incubation with a rabbit anti-mouse IgG-biotin antibody (Sigma, Taufkirchen, Germany) diluted 1:15,000 for 1.5 h followed by alkaline phosphatase-conjugated streptavidin (Invitrogen, Karlsruhe, Germany) diluted 1:3,000 for 30 min. BCIP/NBT (BioRad, München, Germany) was used for the color reaction. For analysis of IgE-reactivity, immunoblots were prepared as described above, except that 20 µg protein extract of WT or transgenic carrots were applied on SDS-PAGE. The blots were cut into strips and incubated overnight with 1:7.5 diluted sera from the carrot-allergic patients and from a non-allergic patient as negative control. Bound IgE antibodies were detected with an alkaline phosphatase-conjugated mouse-anti-human IgE antibody diluted 1:750 for 4 h (Pharmingen, San Diego, CA) using BCIP/NBT as substrate (BioRad).

Skin prick test

SPT was performed according to the method of Dreborg (1989). Approval of 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.

Control SPT solutions (histamine hydrogen chloride 0.1% and sodium chloride 0.9%) as well as carrot and pollen extracts for routine testing (Supplementary Table S1) were purchased from Allergopharma (Reinbek, Germany).

Fresh WT and transgenic carrots were used for prick-to-prick tests. Five crops of each line were tested. Readings were done after 20 min. The mean wheal diameters and the average thereof for each line were determined.

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Figure S 1 A and B

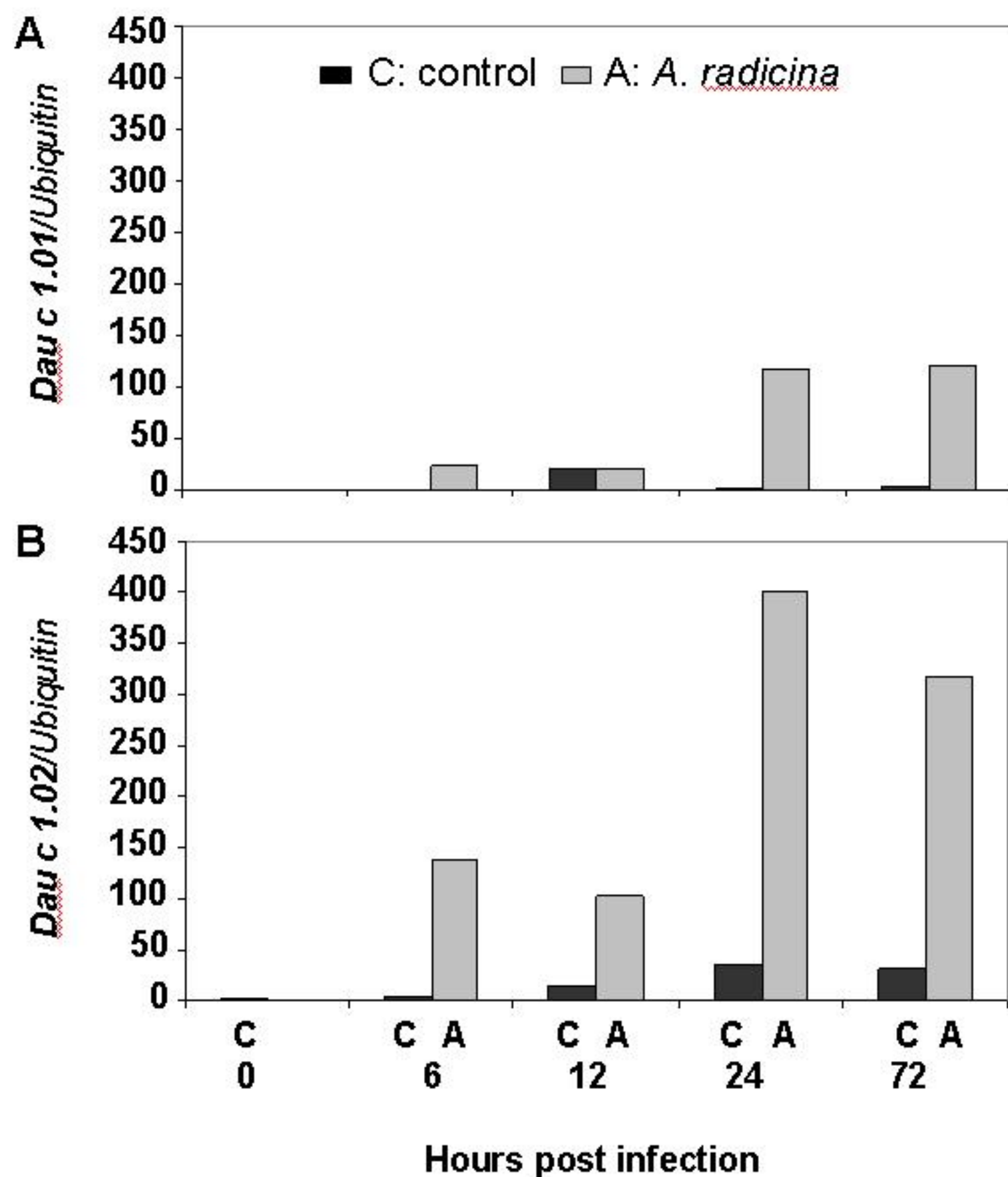


Fig. S 1:

Accumulation of Dau c 1 mRNAs (synonymic DcPR10) in field grown carrots upon challenge with the fungal pathogen *Alternaria radicina*. Slices (0.5 cm thick) from the middle part of 4-month-old fresh roots of the cultivar Rodelika were placed on moist filter paper in a petridish. The slices were inoculated with 10^5 conidia (10 μ l aqueous suspension) and incubated at RT in the dark. Transcripts of Dau c 1.01 (A) and Dau c 1.02 (B) were determined by qPCR at the indicated time points. Values are given relative the ubiquitin RNA.

Figure S 2



Fig. S 2:

Schematic representation of the T-DNA region inserted into plant transformation RNAi vector pK7GWIWG2(II). A 253 nt fragment of Dau c 1.01 (A) and 267 nt fragment of Dau c 1.02 (B) were inserted separately in sense and anti sense orientation into vector pK7GWIWG2(II), resulting in plasmids pK7GW_Dau c 1.01_RNAi and pK7GW_Dau c 1.02_RNAi.

Table S 1:

Clinical and serologic characterization of patients with carrot allergy. (OAS = oral allergy syndrome. ImmunoCAP classes and kilounits per liter (kUA/L) are related as follows: <0.35 kUA/L = class 0; 0.35 to ≤ 0.7 kUA/L = class 1; >0.7 to ≤ 3.5 kUA/L = class 2; >3.5 to ≤ 17.5 kUA/L = class 3; >17.5 to ≤ 50 kUA/L = class 4; >50 to ≤ 100 kUA/L = class 5; >100 kUA/L = class 6.).

TABLE S 1

No.	profession	Past medical history: Further allergies (other than carrot)	clinical symptoms (carrot)	Oral food challenge (carrot)	Routine Skin Prick Test		ImmunoCAP [kU _A /L] (CAP class)				experimental ImmunoCAP [kU _A /L] (CAP class)		
					carrot	birch pollen (Allergo-pharma)	carrot	rBet v 1	rBet v 2	Bromelin	rDau c 1.0104	rDau c 1.0201	rDau c 4
1	travel agent	Seasonal allergic rhinoconjunctivitis (march to june) with beginning asthmatic symptoms. Diagnosed with type I allergies to birch-, weed- and grass-pollen, moulds. OAS and contact urticaria to apple.	OAS and contact urticaria	pos	pos	pos	0.10 (0)	6.62 (3)	0.01 (0)	0.03 (0)	0.00 (0)	2.04 (2)	0.01 (0)
2	medical secretary	Seasonal allergic rhinoconjunctivitis (february to september). Diagnosed with type I allergies to birch- and grass-pollen, moulds, OAS to apple and orange.	OAS	pos	pos	pos	0.07 (0)	43.1 (4)	7.46 (3)	0.28 (0)	5.25 (3)	4.32 (3)	2.11 (2)
3	consulting engineer	Seasonal allergic rhinoconjunctivitis (march to may). Diagnosed with type I allergies to birch-pollen. Severe type I allergy to bee and yellow jacket venom (anaphylactic shock reaction)	OAS	pos	pos	pos	2.82 (2)	35.9 (4)	0.03 (0)	0.88 (2)	3.7 (3)	4.32 (3)	0.09 (0)
4	field engineer	Seasonal allergic rhinoconjunctivitis (march to june) with beginning asthmatic symptoms. Diagnosed with type I allergy to birch-pollen. OAS to apple, celery, hazelnut, peanut. type I allergies (allergic asthma) to cat-, horse- and dog dander. Severe type I allergy to yellow jacket venom (anaphylactic shock reaction)	OAS	pos	pos	pos	5.20 (3)	55.8 (5)	2.7 (2)	0.18 (0)	10.5 (3)	4.9 (3)	1.48 (2)
5	physician	Seasonal allergic rhinoconjunctivitis (march to june) with beginning asthmatic symptoms. Diagnosed with type I allergy to birch-pollen.	OAS	pos	pos	pos	2.20 (2)	9.28 (3)	1.30 (2)	0.02 (0)	2.53 (2)	1.55 (2)	0.88 (2)
6	physician	Seasonal allergic rhinoconjunctivitis (march to june) with beginning asthmatic symptoms. Diagnosed with type I allergy to birch-pollen. OAS to apple, hazelnut and cherries.	OAS	pos	pos	nd	0.62 (1)	5.35 (3)	0.00 (0)	0.08 (0)	0.64 (1)	0.91 (2)	0.05 (0)
7	university student	Seasonal allergic rhinoconjunctivitis (february to june) with beginning asthmatic symptoms. Diagnosed with type I allergy to birch-pollen. Atopic dermatitis.	OAS	pos	pos	nd	1.43 (2)	>100 (6)	0.08 (0)	0.21 (0)	12 (3)	0.77 (2)	0.09 (0)
8	physician	Seasonal allergic rhinoconjunctivitis (march to june). With beginning asmatic symptoms. Diagnosed with type I allergies to birch-pollen.	OAS	pos	pos	pos	1.05 (2)	10.5 (3)	0.00 (0)	0.07 (0)	0.69 (1)	1.06 (2)	0.04 (0)
9	Interior decorator	Seasonal allergic rhinoconjunctivitis (february to may). Diagnosed with type I allergies to birch-pollen. OAS to apple, pear, hazelnut and tomato.	OAS	pos	pos	pos	24.0 (4)	>100 (6)	0.13 (0)	2.90 (2)	28.4 (4)	16.6 (3)	0.54 (1)
10	physician	Seasonal allergic rhinoconjunctivitis (february to april) Diagnosed with type I allergy to birch- and mugwort-pollen.	OAS	nd	pos	pos	8.16 (3)	0.06 (0)	0.14 (0)	0.36 (1)	4.86 (3)	14.4 (3)	0.18 (0)