

Gene transcription analysis of carrot allergens by relative quantification with single and duplex reverse transcription real-time PCR

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Abstract Single and duplex real-time polymerase chain reaction (PCR) systems have been developed to quantify specific mRNA transcription of genes coding for the major *Daucus carota* allergen isoforms Dau c 1.01 and Dau c 1.02. Methods were tested with samples from the local market. Whereas the gene transcription levels for Dau c 1.01 were consistently high in all investigated samples, significant differences for the Dau c 1.02 transcription could be demonstrated in randomly collected market samples. The gene transcription level for the minor Dau c 1.02 variant is about one log below Dau c 1.01. Both formats, single or duplex real-time methods, exhibit ideal

cycle threshold (CT) ranges and good reproducibility. In particular, the easily performed duplex real-time PCR system is potentially suited for the selection of hypoallergenic varieties and studying the impact of post-harvesting or environmental conditions.

Keywords Reverse transcriptase · Real-time PCR · *Daucus carota* · Allergens · Dau c 1.01 · Dau c 1.02 · PCR · Gene expression

Introduction

Antigenic cross-reactivity between carrot, celery, fennel, dill, parsley and birch, mugwort, stone fruits, apple and soybean is the reason for a high incidence of allergic reaction against vegetables and herbal spices of the *Apiaceae* plant family [1–3]. Up to 25% of food-allergic patients are affected by carrots according to the results of a Swiss study [4]. Together with apple, hazelnut, soybean, kiwi and wheat, carrot is most frequently responsible for allergic syndromes as reported for the Berlin population [5]. In contrast to e.g. peanut or soybean, carrots normally provoke rather mild to moderate than severe symptoms after ingestion. In most cases, spontaneous acute oropharyngeal symptoms are attenuated by cooking. Severe adverse effects have been reported in only rare case studies in Southern Europe [6–9]. However, recent scientific evidence indicates that in sensitised patients the prospect to worsen delayed atopic eczema increases significantly even by the contact with the cooked vegetable [10]. Positive reaction with carrot extracts in skin prick testing [5] as well as contact dermatitis from raw carrots is

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Table 1 Gene bank accession numbers

Number	Gene	NCBI accession number
1	Dau c 1.0101	U47087
2	Dau c 1.0102	D88388
3	Dau c 1.0103	Z81361
4	Dau c 1.0104	Z81362
5	Dau c 1.0105	Z84376
6	Dau c 1.0201	AF456481
7	GAPDH	U44985
8	Actin	X17526
9	Ubiquitin	U44983
10	Tubulin	X16608
11	Ribosomal Protein S10	AF287338
12	Ribosomal Protein S15a	AF349962

observed frequently [11] and belongs to the earliest description of skin irritation through food [12]. The relevance of allergy against carrots, being a vegetable of great economic significance, might have been underestimated in the past regarding the potential for allergenic response.

The major carrot allergen Dau c 1 that is associated to the major birch pollen allergen, Bet v 1, is a protein of about 17 kDa size and belongs to the so-called family of pathogenesis related proteins (PR-10). The Bet v 1 gene belongs to a multigene family, and more than 30 isoforms

and additional variants of the European white birch (*Betula pendula* L.; syn. *Betula verrucosa*) cultivars have been described [13]. The gene shares approximately 61% and 93% sequence homology, respectively, with the major birch and celery allergens Bet v 1 and Api g 1. Two isoforms of Dau c 1, namely Dau c 1.01 and Dau c 1.02, are known. Five variants (1.0101–1.0105) of Dau c 1.01 have been described [14–17].

For the detection and quantification of allergenic proteins, immunochemical methods such as the enzyme-linked immunosorbent assay (ELISA) are the first choice. However, ELISA techniques depend on highly specific and sensitive antibodies which are not always commercially available for the target of interest. Because the preparation and validation of non-commercial antibodies is a time-consuming task, the investigation of the mRNA expression of allergen genes might be an interesting alternative tool to estimate the allergenic potential of plant varieties and cultivars. The direct correlation between gene transcription and protein expression is a prerequisite to evaluate the potential of real-time reverse transcriptase (RT) polymerase chain reaction (PCR) as an alternative method in comparison to ELISA. Hence, the development of real-time RT-PCR methods for allergens is a first step towards this direction. Primers and probes used in real-time PCR are highly specific, of reproducible chemistry and low in cost and available to any laboratory within shortest time. The potential for standardisation of such an approach and the

Table 2 Primer and probes for allergen genes

Target/system	Primerpair and probe		Sequence	Bp ^b
Dau c 1.0101 (Consensus 1.0101–10105)	Forward primer	Dau c 1.0101 f	5'-GAGCTTACAAGAGTGTCTG-3'	217
	Reverse primer	Dau c 1.0101 r	5'-GTCAGCAGTTGGCACA-3'	
	Probe	Dau c 1.01 p	5'-FAM-CCTTGTTTACTGCAT CAGTYCTA ACCGTCA-TMR	
Dau c 1.0201 (Sequencing primerpair)	Forward primer	Dau c 1.0201 f	5'-GACACTGTTTTCCCAAGGTTC-3'	242
	Reverse primer	Dau c 1.02-I r	5'-GTTGGCACAACAACGAATTGAT-3'	
Dau c 1.01/(Consensus 1.0101– 10105) (for use in single and duplexPCR)	Forward primer	Dau c 1.01 f	5'-CCTTCCCGAAGGTAGCC-3'	133
	Reverse primer	Dau c 1.01 r	5'-ATGGGTTTCAATGGATTCGAT-3'	
	Probe	Dau c 1.01 p	5'-FAM-CCTTGTTTACTGCAT CAGTYCT AACCGTCA-TMR 5'-YAK-CCTTGTTTACTGCAT CAGTYC TAACCGTCA-TMR ^a	
Dau c 1.02/System I (for use in singlePCR)	Forward primer	Dau c 1.02 f	5'-CATCTTGGCGAAGCAACTGAATA-3'	151
	Reverse primer	Dau c 1.02-I r	5'-GTTGGCACAACAACGAATTGAT-3'	
	Probe	Dau c 1.02 p	5'-FAM-ACCTACACCACCATCGGA GGTG ACAT-TMR	
Dau c 1.02/System II (for use in duplexPCR)	Forward primer	Dau c 1.02 f	5'-CATCTTGGCGAAGCAACTGAATA-3'	214
	Reverse primer	Dau c 1.02-II r	5'-GGGAGCACAGCATCGCCT TT-3'	
	Probe	Dau c 1.02 p	5'-YAK-ACCTACACCACCATCGGA GGT GACAT-TMR	

^a Reporter dye Yakuma Yellow (YAK) in multiplex systems. All probes TAMRA quenched

^b Size of PCR product with cDNA

independency in purchasing the individual reaction components from different suppliers might be a significant advantage over immunochemistry using antibodies from a specific provider.

The aim of our study was to develop and validate single and duplex one- and two-step reverse transcriptase real-time PCR methods to determine the relative transcription level of the genes encoding the isoforms Dau c 1.01 and Dau c 1.02 of the major allergen Dau c 1 in *Daucus carota*. Relative gene quantification is obtained versus a housekeeping gene. The applicability of the RT-PCR methods described was investigated in various carrot samples collected randomly from the market and from ongoing cultivation studies.

Materials and methods

Material

Fresh carrots of known variety from the fields (*Nerac* or *Rodelika*) or from the shelf (*Narbonne*) were used for development and validation of the RT-PCR. The variety

Nerac was arbitrarily chosen as a calibrant in comparative experiments with market samples. Twenty healthy carrots were taken randomly and bundled to provide a representative sample from field material. Methods were tested with pooled samples of ten carrots from each shelf sample collected from the local markets. For carrots derived from the market, no variety indication was available. Selection of the housekeeping gene (HKG) was done with three different carrot tissues.

Sample preparation

Within 48 h after harvesting or purchase, samples were processed in the laboratory. If necessary, leaves were plucked from each bundle, and the whole mixed samples (20 from fields and ten carrots from the market) were chopped roughly to slices about 3 mm thickness using a kitchen mill. Slices were quickly shock frozen by liquid nitrogen to stop any metabolism and stored at -80°C until extraction. Frozen samples were ground to powder (Grindomix, Retsch, Haan, Germany) under liquid nitrogen. Subsequently, the frozen powder was divided and stored at -80°C until extraction.

Table 3 Primer and probes for housekeeping genes

Target/system	Primerpair and probe		Sequence	Bp
GAPDH(glycerine aldehyde phosphate dehydrogenase)	Forward primer	Dau c-GAPDH-f5	5'-GTGACAGCAGGTCGAGCATC-3'	113
	Reverse primer	Dau c-GAPDH-r	5'-CAACCACTCGGGTGCTGTAT-3'	
	Probe	Dau c-GAPDH-p	5'-FAM-CCCATTTCATTGTCGT ACCACGAAACAA-TMR 5'-YAK-CCCATTTCATTGTCGT ACCACGAAACAA-TMR ^a	
Actin (Carrot)	Forward primer	Dau c Ac f	5'-AAAGGATGCCTATGTTGGT-3'	140
	Reverse primer	Dau c Ac r	5'-CACGCAGCTCATTGTAGAA-3'	
	Probe	Dau c Ac p	5'-FAM-AGGGGTATCATCACATTG AAATA-TMR	
Ribosomal Protein S15a-1	Forward primer	S15a S	5'-GCTGGCATCATGGATCACG-3'	116
	Reverse primer	S15a A	5'-CAAAGGGTACAAGAAAATTGG AATC-3'	
	Probe	—	—	
Ribosomal Protein S15a-2	Forward primer	Dau c S15a f	5'-TCAAGACAGTTTGGTTATATCGTG C-3'	156
	Reverse primer	Dau c S15a r	5'-AATCAAGCACTAAGCCAGTTATCC A-3'	
	Probe	—	—	
Ubiquitin	Forward primer	Dau c UBI f	5'-AGA GACCTACACCAAGCCCAAGA-3'	85
	Reverse primer	Dau c UBI r	5'-CTCATCCACCTTATAGAACTGC-3'	
	Probe	—	—	
Tubulin	Forward primer	Dau c TUB f	5'-GAATACCAGCAGTACCAAGA-3'	89
	Reverse primer	Dau c TUB r	5'-CATTACATATCTTGATGAGCC-3'	
	Probe	—	—	
Ribosomal Protein S10	Forward primer	Dau c RIBS10-f	5'-TGAAATTGAAAGTGTGGGACT-3'	117
	Reverse primer	Dau c RIBS10-r	5'-ACAACCTCAGGAACCGCAACC-3'	
	Probe	—	—	

^a Reporter dye Yakuma Yellow (YAK) in multiplex systems. All probes TAMRA quenched;

Isolation of RNA, determination of nucleic acid concentration and quality

From each sample of frozen carrot powder, aliquots of 100–200 mg were taken to gain two to three independent extractions. RNA was extracted with the Pure-Link™ Micro-to-Midi™ total RNA Purification System (Invitrogen, Life Technologies Corp, Carlsbad, USA) following the TRIzol® Plus RNA Purification protocol given in the manual. To remove DNA from RNA preparations, a 5-min DNase digestion (RNase-Free DNase Set, Qiagen GmbH, Hilden, Germany) was carried out. Per sample, 30 µl RNA was eluted from the spin columns with RNase-free water (Fluka, Sigma-Aldrich Inc., St. Louis, USA). The amount of isolated RNA was measured spectrophotometrically using a NanoDrop® ND-1000 (Nanodrop Technologies, Inc.,

Fisher Scientific, Germany) device. RNA preparations yielded concentrations of about 50–220 ng/µl with 260/280 nm absorbance ratios between 1.8 and 2.2 demonstrating clean nucleic acids.

Reverse transcription

Purified RNA was transcribed in a two-step reaction into cDNA prior to PCR using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) and Oligo dT(20) primer according to manufacturer's instruction. RNA (400 to 1,000 ng) was used for the reaction. Two microlitres reverse-transcribed RNA (containing 10–100 ng/µl cDNA depending on experiment) was used in PCR as described below. For duplex PCR, a one-step procedure was chosen carrying out reverse transcriptase and PCR subsequently in one tube with the 2×

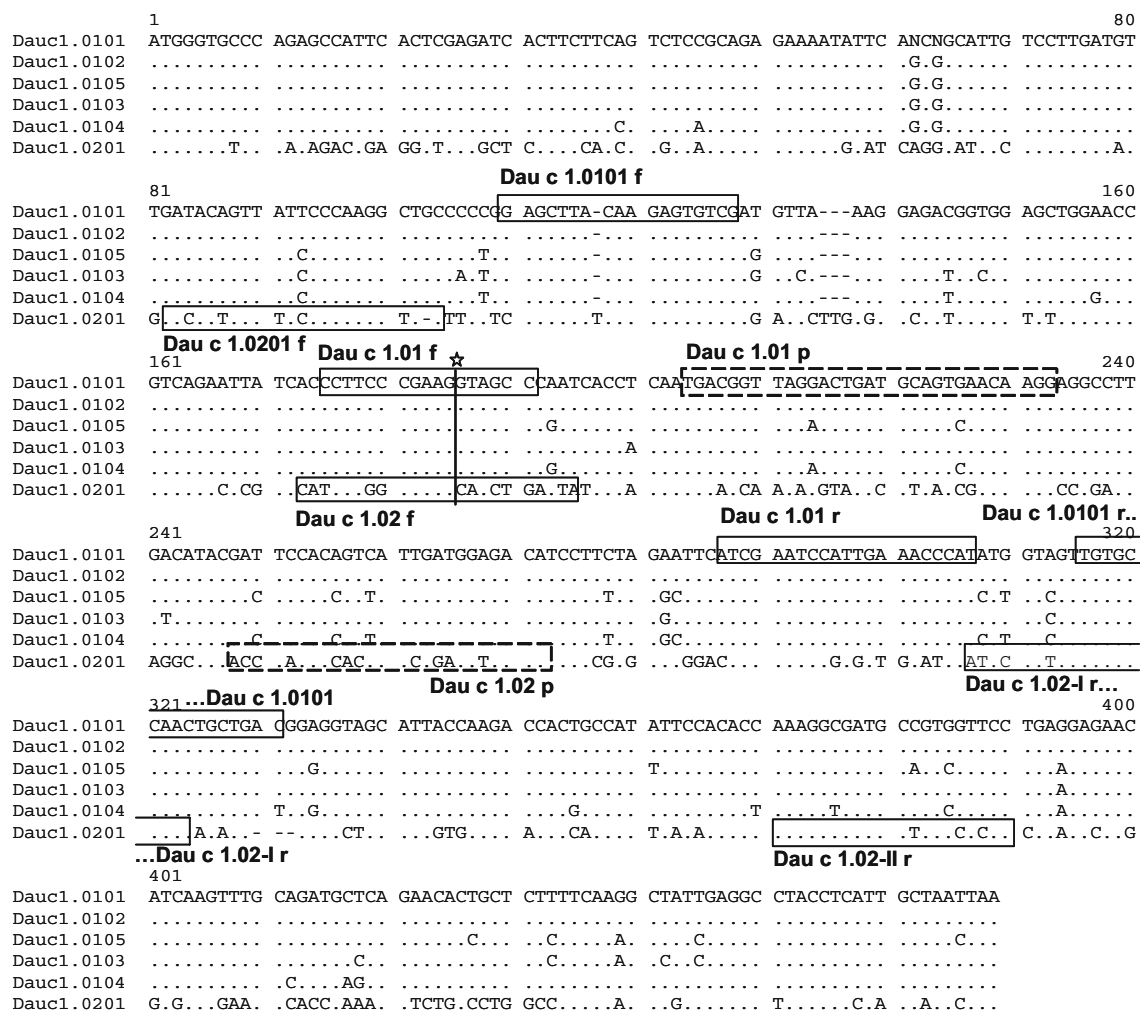


Fig. 1 Sequence alignment for the allergen variants Dau c 1.0101–1.0105 and Dau c 1.0201. The position of oligonucleotides in the consensus region is indicated by continuous (primers) or dashed

(probes) frames. The splice site at codon position 62 of the related birch PR10 protein is indicated by an asterisk

QuantiTect® multiplex RT-PCR-Kit-inclusive ROX (Qiagen GmbH) following the kit description (see section “Duplex real-time PCR”).

PCR (primer and probes)

All PCR primers for the identification of unpublished intron sequences were designed on the basis of known sequences and sequence stretches from NCBI GenBank sequence data (Table 1). Amplified PCR products carrying intron stretches were DNA sequenced (AGOWA GmbH, Berlin). For the design of primers and probes for the sequence-specific amplification of the Dau c 1.01 and Dau c 1.02 transcripts, sequence information of the intron and exon positions were used, to discriminate between genomic DNA and cDNA. Primer and probe sequences are listed in Tables 2 and 3. All primers and probes were synthesised by TIB MOLBIOL GmbH (Berlin).

PCR conditions

Classical PCR

For classical PCR, the following reagents were pipetted per reaction: 3.75 mM MgCl₂ buffer and 0.2 mM each dNTP (Applied Biosystems, Roche), 0.3 μM of each primer forward and reverse, 1.5 U HotStar-Taq polymerase (Qiagen) and water to a volume of 18 μl. Two microlitres of template cDNA (50 ng) was added per reaction. PCR was performed in an ABI 9700 (Applied Biosystems) device. The standard time and temperature programme which was applied for all systems consisted of an initial denaturation step (12 min, 95°C) followed by 40 cycles 30 s denaturation at 95°C, 30 s annealing at 60°C, 60 s extension at 72°C and 7 min final elongation at 72°C.

For analysis of PCR products, 10 μl of the reaction was mixed with 2 μl loading buffer (Xylencyanol-Saccharose buffer, Merck KGaA), and subsequently, 10 μl was applied per slot. PCR products were separated on 1.5% agarose gels in 0.5× TBE buffer (Tris-Borat 45 mM–Na₂-EDTA-10× buffer, pH 8.0–8.3, Merck) at 300 V/150 mA/1 min and 150 V/150 mA/46 min. The product size was estimated by comparison with a 100-bp standard ladder (Marker XIV, Roche Diagnostics GmbH).

Real-time PCR

Singleplex real-time PCR

For the singleplex (sp) real-time PCR systems spDau c 1.01, spDau c 1.02 and spGAPDH used for system development, the following components were mixed per

reaction: Universal Mastermix (Eurogentec, RT-QP2X-03-075+, including uracil-*N*-glycosylase (UNG) and Hot-GoldStar polymerase) 1-fold, 0.3 μM of each primer forward and reverse, 0.2 μM FAM-labelled probe and water to a volume of 18 μl. Two microlitres template DNA (50 ng in case of reverse-transcribed RNA) was added per reaction. For testing primer performance, 10-fold dilution series between 100 ng and 0.001 ng were prepared and amplified with a SYBRgreen mastermix. Real-time PCR was performed on an ABI 7900HT (Applied Biosystems) thermocycler using the following standard time–temperature programme: UNG activation 2 min 50°C, denaturation 10 min 95°C, followed by 40 cycles denaturation 15 s 95°C, annealing and extension 60 s 60°C. Baseline and thresholds were processed automatically by SDS 2.2.1 software.

Duplex real-time PCR

Duplex (dp) one-step PCR was performed using the reverse transcription (rt) and PCR mastermix system contained in the 2× QuantiTect® multiplex RT-PCR-Kit (including ROX, Qiagen GmbH). Reverse transcription and PCR with

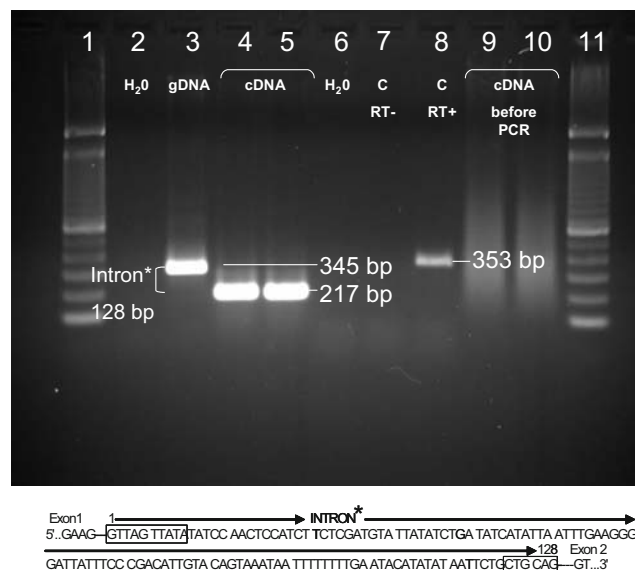


Fig. 2 Gel electrophoresis of PCR products generated with the primer pair 1.0101 f/10101 r (see Table 2) and the consensus PCR system Dau c 1.0101 applied on cDNA following reverse transcription of carrot RNA. Lanes 1 and 11 size standard (100 bp ladder); lanes 2 and 6 blank (water instead of cDNA input in PCR); lanes 4 and 5 PCR product obtained with carrot cDNA, two extractions; lane 3 PCR product obtained with genomic carrot DNA; lanes 7–10 reverse transcription control: RT performed without (RT–) and with (RT+) 1 μl total HeLa RNA (100 pg/μl) input in RT reaction. In case of successful reverse transcription, a 353-bp product is generated in PCR with human actin control primers (delivered with kit)

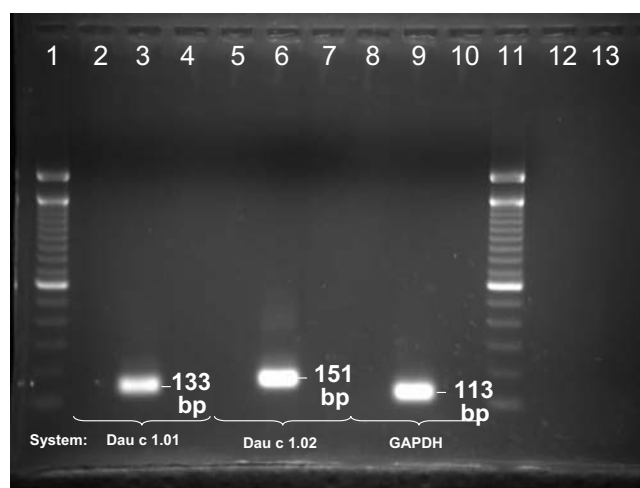


Fig. 3 Gel electrophoresis of PCR products generated with 10 ng input cDNA or DNA derived from carrot market samples of unknown variety using different primer pair combinations. Lanes 1 and 11 size standard (100 bp ladder), lanes 2, 5 and 8 blank (water instead cDNA input in PCR), lanes 3 and 4 PCR product with primer pair Dau c 1.01 f/Dau c 1.01 r (system spDau c 1.01) and carrot cDNA (lane 3) or DNA (lane 4), lanes 6 and 7 PCR product with primer pair Dau c 1.02 f/Dau c 1.02-I r (system spDau c 1.02) and carrot cDNA (lane 6) or DNA (lane 7), lanes 9 and 10 PCR product with primer pair Dau c-GAPDH-f/Dau c-GAPDH-r (system GAPDH) and carrot cDNA (lane 9) or DNA (lane 10), lanes 12 and 13 empty. Because all primer systems span a splice site, PCR products only appear with cDNA

TaqMan probes in a mastermix was performed according to the manual instructions for quantitative real-time duplex one-step RT-PCR. The optimal primer concentration for the duplex system dpDau c 1.01/glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) as determined by primer titration was 0.3 μM ; the primer concentration for the duplex system dpDau c 1.02 II/GAPDH was 0.9 μM each. The concentration of the Yakuma Yellow-labelled GAPDH probe and the FAM-labelled allergen gene specific probe was 0.2 μM each. One-step reverse transcription and real-time PCR

were run in an ABI 7900HT (Applied Biosystems) thermocycler using the following standard programme: 20 min 50°C reverse transcriptase, 15 min 95°C initial denaturation, 40 cycles 45 s 95°C denaturation and 45 s 60°C annealing and extension. Baseline and thresholds were processed automatically by SDS 2.2.1 software.

Determination of relative expression levels

Relative gene expression levels were determined on basis of the $\Delta\Delta\text{CT}$ Method according to Livak and Schmittgen (2001) [18]. CT values obtained for the Dau c 1.01 and Dau c 1.02 gene were first normalised for each sample respectively to the endogenous housekeeping gene (see “Results and discussion”; $\text{CT}_{\text{Target}} - \text{CT}_{\text{HKG}} = \Delta\text{CT}$). Subsequently, the obtained ΔCT value is normalised to an external calibrator ($\Delta\text{CT} - \text{CT}_{\text{cal}} = \Delta\Delta\text{CT}$). A pooled field sample of the variety Nerac arbitrarily served as calibrator sample. Relative expression levels are calculated according to the formula $2^{-\Delta\Delta\text{CT}}$ [18].

Results and discussion

Primer development

Comparison of published gene sequences for the five known variants of the allergen isoform Dau c 1.01 (Dau c 1.0101–1.0105) discloses a region of high sequence homology (Fig. 1). A splice intron site (AGGT) was identified by sequencing the product obtained with the consensus primer pair Dau c 1.0101 f (forward)/Dau c 1.0101 r (reverse). The primer pair amplifies different-sized PCR products of 345 and 217 bp, respectively, depending on whether genomic DNA or reversed transcribed RNA (cDNA) was used in PCR (Fig. 2). The spliced 128-bp

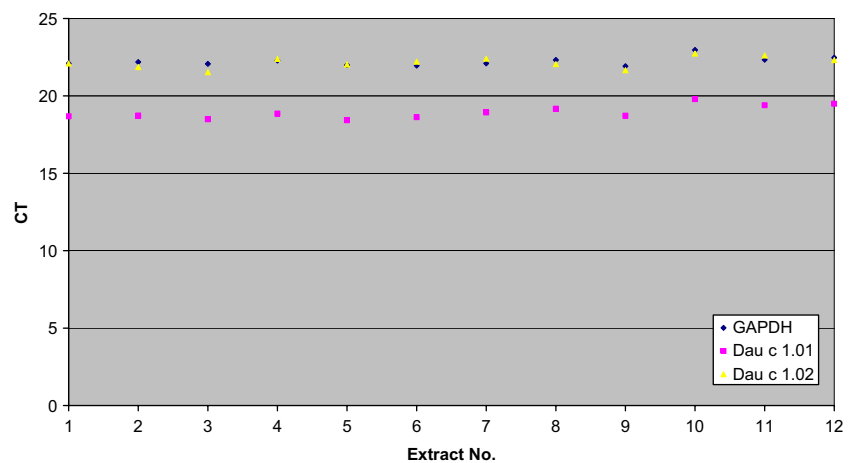
Table 4 Validation of housekeeping genes—average variation of CT values determined with two individual samples and three separated tissue types of carrot root

$n=12^a$	GAPDH	UBI	S15a(1)	S15a(2)	Tubulin	Actin	RibProtS10
geo Mean CT	23.16	24.24	25.41	25.33	26.23	26.12	29.32
ar Mean CT	23.17	24.25	25.42	25.34	26.23	26.12	29.33
Min CT	21.69	23.22	24.51	24.39	25.71	25.65	28.5
Max CT	23.96	24.95	26.5	26.46	26.72	26.83	30.45
SD $\pm$ CT	0.58	0.35	0.45	0.48	0.28	0.33	0.5
CV (%CT)	2.48	1.43	1.77	1.9	1.05	1.25	1.71
Effic. mean	1.00	1.02	1.04	1.02	1.01	1.00	1.18

geo Mean geometric mean, ar Mean arithmetic mean, Effic. efficiency $E^{-1/\text{slope}} - 1$

^a Average CT values derived from two different carrots of the variety *Narbonne*. From each sample, three different tissues (epidermis, parenchyma and central root) were separated to yield subsamples, and from each subsample, two independent extractions (total $n=12$) were measured in double replicates

Fig. 4 CT values obtained with the singleplex systems spDau c 1.01, spDau c 1.02 (10 ng cDNA/reaction) in comparison with spGAPDH. The diagram shows the result achieved with $n=12$ *rt*-RNA preparations (four mixed samples, three independent extractions) gained from the carrot variety Rodelika. Measurement points are mean values from three replicates



intron is flanked by a typical eucaryotic intron 5'-GT start and 3'-AG end. The starting motif of the intron, 5'-GTTAGTTA, as well as the closing motif at the 3'-end, CTGCAG (Fig. 2), is homologous to intron sequences published for the allergens Bet v 1, Cor a 1 and Mal d 1 in birch, hazel and apple which belong to class 10 of the pathogen-related protein family (PR-10) [19]. For the isoform Dau c 1.02, the primer pair 1.0201 f/1.0201 r confirmed a splice site at the same position, but the sequenced intron is much larger covering 626 bp (data not shown).

The knowledge of these splice intron sites accounts for the avoidance of measuring genomic contaminations as we could design primers which were laid across two neighbored exons.

For singleplex real-time PCR, the primer pair Dau c 1.01 f/Dau c 1.01 r and the probe Dau c 1.01 p were selected to specifically amplify a consensus sequence of 133 bp size of the variants Dau c 1.0101–1.0105. The primer pair and probe system Dau c 1.02 f/Dau c 1.02-I r and probe Dau c 1.02 p, which was used for specific detection of the Dau c 1.02 isoform, generates a 151-bp PCR product (Fig. 3). All primer and probe positions used in this study are depicted in Fig. 1.

Housekeeping gene selection

Importance was attached to the selection of a robust and reliable housekeeping gene which is constitutively and equally expressed in all tissue types of the carrot root. Therefore, different HKG candidate real-time PCR systems were generated based on published sequences (Table 1) of GAPDH, actin, ubiquitin, tubulin, ribosomal protein S10 and ribosomal protein S15 (two systems). Real-time PCR was performed with identical input amounts of reverse-transcribed RNA isolated from peel, parenchyma and central tissue. The tissue subfractions were taken from two different individuals of the

variety Narbonne. For all HKG, the tissue type had no significant influence on the average CT values shown by close geometric and arithmetic mean values and low standard deviations in repeated experiments. Mean

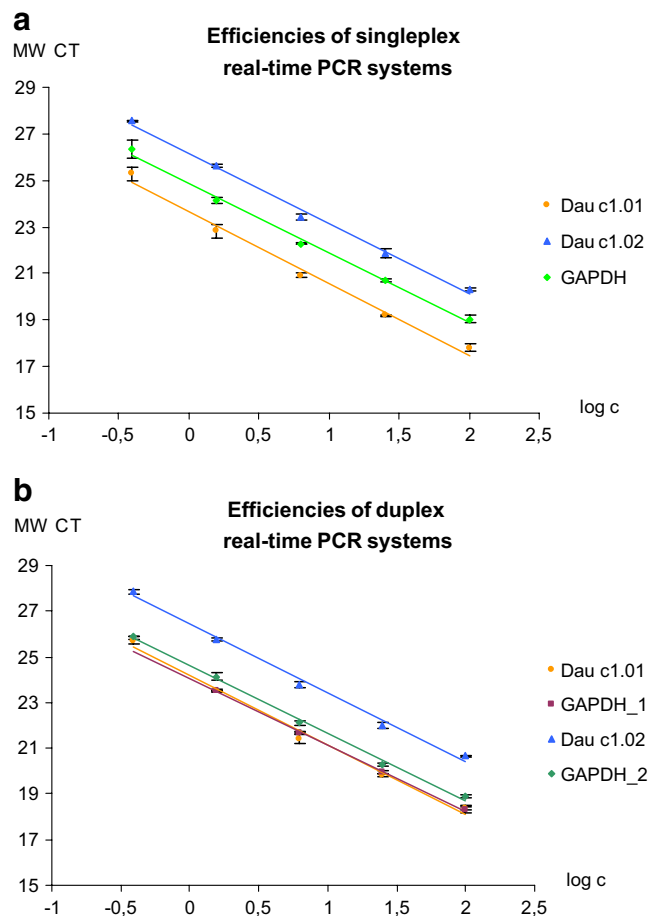


Fig. 5 a, b Resulting linear graphs obtained with the duplex and single PCR systems Dau c 1.01, Dau c 1.02 and GAPDH and diluted transcribed RNA from carrot market samples. Comparable efficiencies can be observed irrespective if the systems are run in single tube or HKG and allergen-specific PCR together in one duplex format

Table 5 Linear equation derived from CT values (mean value of two independent extractions in double determination) plotted against the log of input cDNA concentration from market sample of carrots

	Linear equation	Efficiency $[10^{-1/\text{slope}} - 1]$
Singleplex		
Dau c1.01	$Y = -3.0819x + 23.655$	1.11
GAPDH_1	$Y = -2.9990x + 24.891$	1.15
Dau c1.02	$Y = -3.0264x + 26.163$	1.14
GAPDH_2	$Y = -2.9608x + 24.617$	1.17
Duplex		
Dau c1.01	$Y = -3.0328x + 24.176$	1.14
GAPDH_1	$Y = -2.8878x + 24.038$	1.22
Dau c1.02	$Y = -3.0211x + 26.427$	1.14
GAPDH_2	$Y = -2.9608x + 24.617$	1.17

Starting amount, 100 ng cDNA; 1:4 dilution series, Quantitect Multiplex PCR kit (Qiagen). Each PCR system measured single or in duplex format HKG + Allergen-specific gene

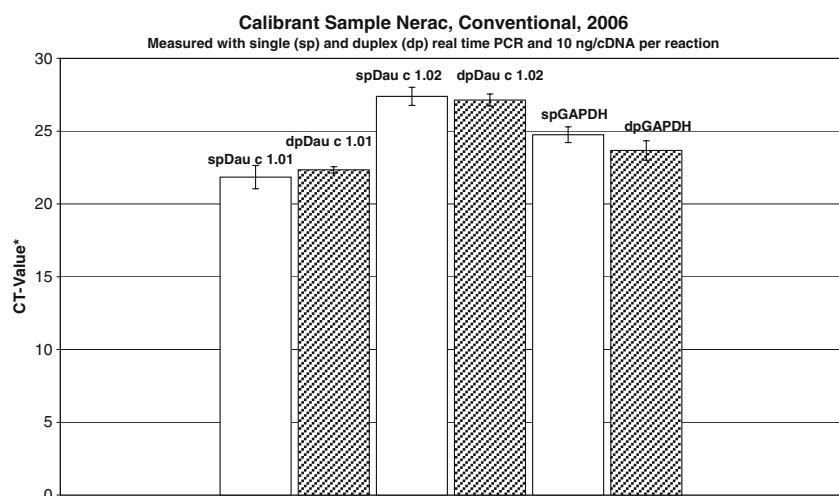
efficiencies of the PCR systems were calculated by the CT slope method. We generated a 10-fold dilution series of the target template and determined the CT value for each dilution. A plot of CT versus log cDNA concentrations was constructed. Valid efficiencies should exhibit a slope of approximately -3.32 . The efficiency is calculated by the formula $[10^{-1/\text{slope}} - 1]$ and should result in a value of about 1. We achieved ideal average values about 2 for all systems and tissues, except for the PCR system targeting the ribosomal protein S10 for which the widest CT range was measured (Table 4). Since this system exhibited also the highest CT values (lowest amplification) in our validation experiment, this HKG was not considered. For our experiments, we finally chose

GAPDH because the system combines good efficiencies and strong target amplification (highest CT values) in the range of the target systems Dau c 1.01 and 1.02. Moreover, the forward primer spans a splice site so that the GAPDH real-time PCR system generates an amplicon of 113 bp size exclusively from cDNA.

Performance of singleplex and duplex PCR reverse transcriptase real-time PCR systems

The singleplex real-time PCR systems spDau c 1.01, spDau c 1.02 and spGAPDH showed comparable mean efficiencies of 1.05, 1.04 and 1.02, respectively, which was evaluated by plotting the CT values against the log of concentration of input cDNA in dilution series experiments (data not shown), with cDNA derived from a sample of the variety Nerac serving as internal reference sample for development and validation of the RT-PCR method. The sensitivity was determined to be 10^{-5} ng/RNA for the spDau c 1.01 real-time PCR system and 10^{-3} ng/(rt)RNA used in spDau c 1.02 real-time PCR, corresponding to a theoretical minimum of ten genome copies for the latter system calculated on basis of a DNA C value of 1 pg (<http://data.kew.org/cvalues/>). As demonstrated with samples derived from the field, both allergen-specific systems are strongly correlated with the HKG GAPDH (Fig. 4).

Based on the singleplex primer systems, duplex PCR formats were developed to perform HKG and allergen gene specific real-time PCR in parallel in one reaction. Cross-amplification between the primers was checked with different primer pair combinations using a representative carrot cDNA sample. The experiments showed that it was not possible to use all three systems in one reaction because of a cross-reaction between the primer Dau c-GAPDH f and Dau c 1.02-1 r (data not shown). Therefore, two duplex

Fig. 6 CT values obtained with 10 ng/cDNA of a calibrant sample (Nerac, conventional, field trial 2006) and single (sp) or duplex (dp) real-time PCR systems. *Asterisk* mean data from 5 mixed carrot samples $\times$ 3 extractions $\times$ 3 replicates ($n=36$)

real-time PCR systems were established. For the duplex dpDau c 1.02 system, the forward primer from the spDau c 1.02 real-time system and a new designed reverse primer Dau c 1.02-II r was used. The resulting amplicon exhibits a size of 214 bp. The dpDau c 1.01 system consists of the same primer and probe combination used in the spDau c 1.01 system but applies another dye for the GAPDH probe

(Yakuma Yellow instead of FAM). The different dyes were necessary to discriminate both PCR products, allergen gene specific and HKG, in one tube. The efficiencies of both duplex systems were calculated on basis of CT values determined in 1:4 dilution series (Fig. 5a, b, Table 5). The data clearly show that the duplex format exhibits similar efficiency than the singleplex format.

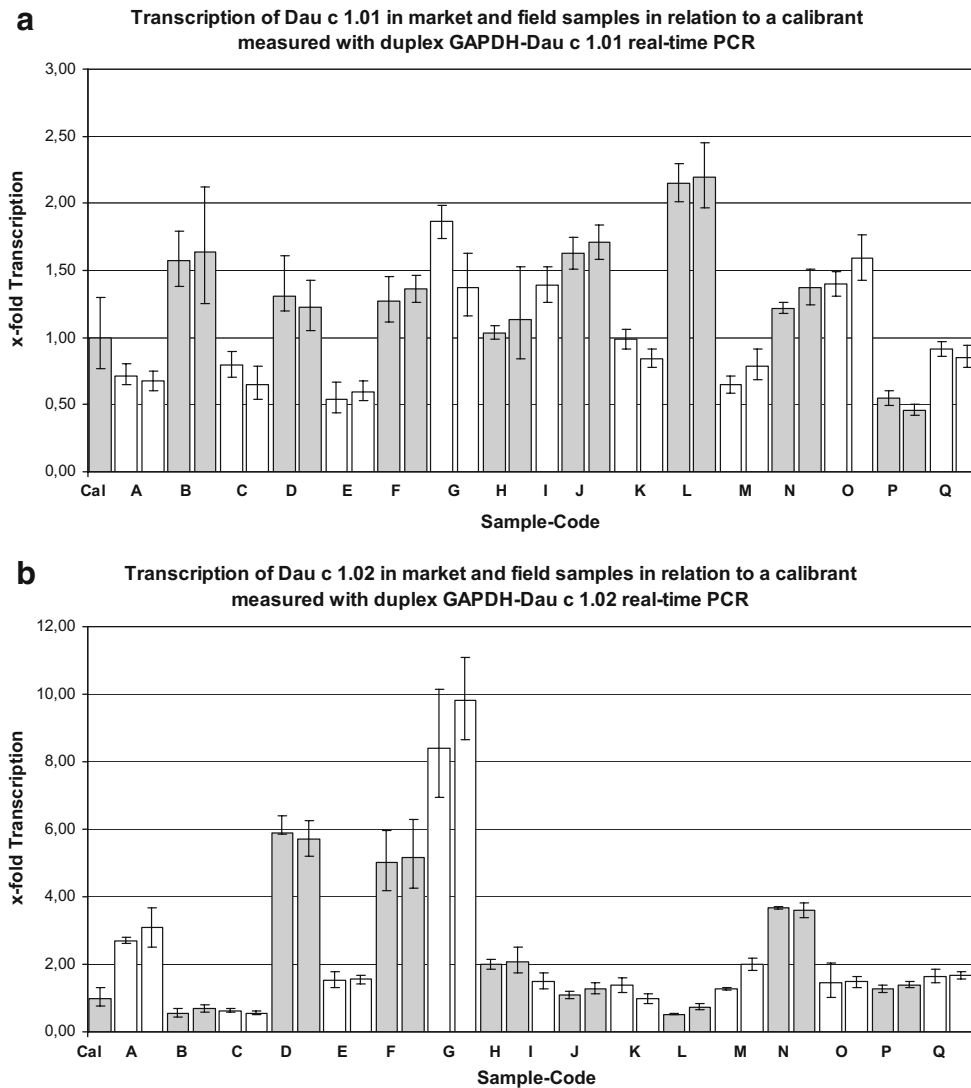


Fig. 7 **a** Transcription level of the allergen gene Dau c 1.01 in random market samples of unknown variety measured with the duplex system dpGAPDH-Dau c 1.01 in relation to an arbitrarily chosen calibrant carrot (Nerac, field trial, conventional farming, crop 2006). Origin and type (organic or conventional) of market samples. *A* market, conv., Germany (GE); *B* market, conv., GE; *C* supermarket, conv., Italy (IT); *D* market, org., GE; *E* market, conv., GE; *F* market, org., GE; *G* supermarket, org., IT; *H* supermarket, org., GE; *I* bio-retailer, GE; *J* market, conv., GE; *K* supermarket, conv., GE; *L* market, conv., GE; *M* market, conv., GE; *N* market, conv., GE; *O* supermarket, org., GE; *P* supermarket, conv., Spain (ES); *Q* Bio-retailer, GE. Except for sample I, two independent RNA extractions were measured (alternating white and grey bars). Error bars exponentiated errors of standard deviations derived from three technical replicates. **b**

Transcription level of the allergen gene Dau c 1.02 in market samples measured with the duplex system dpGAPDH-Dau c 1.02 in relation to an arbitrary chosen calibrant carrot (Nerac, field trial, conventional farming, crop 2006). Origin and type (organic or conventional) of market samples. *A* market, conv., GE; *B* market, conv., GE; *C* supermarket, conv., IT; *D* market, org., GE; *E* market, conv., GE; *F* market, org., GE; *G* supermarket, org., IT; *H* supermarket, org., GE; *I* bio-retailer, GE; *J* market, conv., GE; *K* supermarket, conv., GE; *L* market, conv., GE; *M* market, conv., GE; *N* market, conv., GE; *O* supermarket, org., GE; *P* supermarket, conv., Spain; *Q* Bio-retailer, GE. Except for sample I, two independent RNA extractions were measured (alternating white and grey bars). Error bars exponentiated errors of standard deviations derived from three technical replicates

Calculation based on the $\Delta\Delta CT$ method requires approximately equal efficiencies of the target and reference systems if efficiency correction should be omitted. A sensitive method for assessing if two PCR systems have the same efficiency is to look at how ΔCT values vary with template dilution. If the absolute value is close to zero, the efficiencies of the target and the reference system are similar, and the $\Delta\Delta CT$ method can be used [18]. The relative efficiency plots (delta CT Target–delta CT HKG) for each of our systems results in a slope of <0.1 (data not shown). Therefore, we could perform a valid $\Delta\Delta CT$ analysis with our systems.

Sample analysis

The developed methods were tested with samples randomly collected from the markets and retailer. To compare different samples and PCR systems, a calibrant sample was chosen arbitrarily for all investigations. The calibrant consisted of the carrot variety Nerac cultivated under common conditions. A representative selected cDNA extract from the same Nerac field was measured with the single PCR systems and compared with the duplex PCR system. The CT values achieved with both systems are in a comparable range proving equal performance. Slight non-significant variations can be interpreted due to pipetting and dilution bias (Fig. 6). All market samples were investigated using the duplex assays dpDau c 1.01 and dpDau c 1.02 (Fig. 7a, b). Whereas the transcription level determined for Dau c 1.01 ranges moderately around the calibrant's value (Fig. 7a), divergences up to approximately 10-fold over-expression compared with the calibrant could be observed for the Dau c 1.02 variant (Fig. 7b). Although the highest transcription levels were measured for produce from organic farming (samples D, F and I), the number of investigated samples is too low to draw a conclusion; foremost, two other organic farming samples (O and Q) showed no elevated gene expression. The data demonstrate that, especially for the Dau c 1.02 isoform of the major carrot allergen, differences with respect to the gene transcription level among arbitrarily collected samples can be observed. Both assays, whether simple or duplex format, are well suited for determination of the transcription level of the major carrot allergen Dau c 1.01 and 1.02 genes.

Conclusion

Single and duplex reverse transcriptase real-time PCR systems have been developed and validated to measure the transcription profile of the major *D. carota* allergens Dau c 1.01 and Dau c 1.02 in unknown carrot samples. According to the sequencing data of the PCR amplicons,

the methods demonstrated specificity for either Dau c 1.01 or Dau c 1.02 isoforms. Hence, these novel RT-PCR methods can be used to quantify the transcription levels of the known major allergen sequences of carrot in market samples or to determine the influence of storage and other post-harvesting conditions on the expression of these allergen-coding genes. According to our experience with samples from the market, the fast and simple duplex real-time PCR method, which targets GAPDH and the isoform Dau c 1.02 in one tube, is a promising approach to determine the allergen expression in carrot plants.

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