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Research Article

Identification of four IgE-reactive proteins in raspberry (*Rubus ideaeus* L.)

Gorji Marzban[1], Anita Herndl[1], Daniel Kolarich[2]*, Fatemeh Maghuly[1], Agata Mansfeld[1], Wolfgang Hemmer[3], Hermann Katinger[1] and Margit Laimer[1]

[1] Plant Biotechnology Unit, Department of Biotechnology, BOKU, Vienna, Austria
[2] Glycobiology Division, Department of Chemistry, BOKU, Vienna, Austria
[3] Floridsdorf Allergy Center, Vienna, Austria

IgE-reactive proteins in raspberry (*Rubus ideaus* L.) were identified using PCR, RT-PCR, 2-DE and MS/MS peptide sequencing. Specific polyclonal antibodies and patient sera were used in Western blotting to identify crossreactive epitopes. Initially, two potential allergens Rub i 1 and Rub i 3 were detected using PCR, showing high sequence identity to proteins in Rosaceous species like Mal d 1 and Mal d 3 from apple, Pru av 1 and Pru av 3 from cherry and Pru p 1 and Pru p 3 from peach. Furthermore, *de novo* identified peptides of a protein band at about 30 kDa reacting with most of the patient sera tested (>80%) revealed a high sequence homology with class III chitinases. Raspberry chitinase, when subjected to glycoproteomic analysis, showed typical complex plant-type *N*-glycans with a core α1,3 fucose and a β1,2 xylose at least at one position, indicating the presence of cross-reacting carbohydrate determinants (CCDs). Finally, MS/MS analysis revealed an IgE-reactive raspberry cyclophilin, homologous to Bet v 7. Results obtained suggest that the consumption of raspberries might be responsible for adverse reactions in sensitised individuals.

Keywords: Class III chitinase / Cyclophilin / Rub i 1 / Rub i 3 / Small fruits

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1 Introduction

Raspberries are among the small fruits with promising health benefits due to their high ellagic acid content [1]. However, consumption of fruits may also impose adverse effects in individuals with fruit allergy [2]. Epidemiological data estimate the prevalence of food allergies to be 1.4–2.4% worldwide with an increasing progression [3] and children and young adults being particularly affected [4, 5].

The clinical importance of fruit allergens like Mal d 1, a Bet v 1 homologous protein from apple and non-specific lipid transfer proteins (nsLTPs), has been repeatedly emphasised [6, 7]. Allergic symptoms related to Mal d 1 and homologous proteins are generally reported as mild and local, which is due to the chemical lability of these proteins [8]. Due to the fact that Mal d 1 is degraded during the gastrointestinal transit, it cannot sensitise directly and is claimed to be only capable of triggering allergy in patients previously sensitised by birch pollen allergen Bet v 1. However, when conformational epitopes of Bet v 1 were experimentally stabilised, hypersensitivity could be induced *via* oral route in mice, leading for the first time to the indication that digestion insufficiency is able to support food allergy [9]. Also proton pump inhibitors were shown to promote the development of immediate type food allergy towards digestion-labile proteins in animal model and adult patients [10].

LTPs, on the other hand, are highly stable proteins, containing eight conserved cystein residues forming four disulphide bridges, able to induce anaphylaxis after consumption [11]. Based on protein sequence and structural similarities, these allergens are classified as pathogenesis-related proteins (PRs), suggesting an important role in the plant defence [12]. In this context, their expression profile and mode of action are under extensive investigation, in order to develop novel breeding strategies.

Correspondence: Professor Margit Laimer, Plant Biotechnology Unit, Department of Biotechnology, Muthgasse 18, BOKU, 1190 Vienna, Austria
E-mail: m.laimer@iam.boku.ac.at
Fax: +43-361-3697615

Abbreviations: LTP, lipid transfer protein; **PR,** pathogenesis-related protein

* Present address: Core of Biomolecular Frontiers, Macquarie University, NSW 2109, Sydney, Australia.

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Although inhalation of frozen raspberry powder was reported to cause occupational asthma in adult factory workers, allergens in raspberries have not been characterised so far [13]. An initial analysis of genetic sequences within the *Rosaceae* indicates that coding regions of the most genes are conserved [14]. The high level of synteny among Rosaceous fruits like raspberry and apple, suggests a very similar protein expression pattern [15]. However, proteomic data on fruit proteins are difficult to obtain due to the complexity of the plant specific tissue matrix and the low protein content [16–19].

2-DE in combination with IgE-Western blotting was applied for a selective allergen profiling in raspberries [20]. cDNA sequence analysis has already been shown to be a powerful tool in the characterisation of plant allergens [21]. Additionally inhibition assay must be employed to reconfirm the existence of crossreactive epitopes of potential allergenic proteins. Carbohydrate moieties on the surface of glycoproteins, are widely distributed in plant-derived glycoproteins and represent frequently encountered epitope structures for IgE binding [22]. These glycan chains are reported among the primary causes for crossreactivity and have been designated as crossreacting carbohydrate determinants (CCDs) [23]. In this study, 2-DE Western blots with polyclonal antibodies raised against the major apple allergens and sera from patients allergic to Rosaceous fruits, followed by MS/MS and LC-ESI-Q-TOF MS were applied to characterise IgE-reactive proteins from raspberry.

The current study addressed two main questions: (i) do raspberries share the most common IgE-reactive protein pattern with apples, and express Mal d 1 and Mal d 3 (nsLTP) homologous proteins? (ii) can IgE-reactive raspberry proteins be classified in well-known protein families like PRs or might other proteins be involved in the allergen response?

2 Materials and methods

2.1 Total RNA isolation and RT-PCR analysis

Total RNA was isolated from fresh raspberries obtained from the supermarket with the RNeasy Plant Mini Kit (QIAGEN). RNA quality and concentration were determined by gel electrophoresis and spectrophotometry, respectively. Contaminating DNA was removed with DNase I (Roche).

First strand cDNA was synthesised by RT using about 2.5 μg total RNA, SuperScript™ II RNase H^- RT and oligo (dT) primers (Invitrogen), according to the supplier's recommendation. The RT reaction mix was diluted twice to reduce inhibitory effects of the RT buffer system.

Based on the expected homology between the Rosaceous species, the following primers were used for the detection of the allergens in raspberry fruits: (MD1Fdeg 5′-NNNGAATTCATGGGTGTSTWCACATWYGAA-3′ and MD1Rdeg 5′-NNNGGATCCTTAGTTGTAGGGRTCCK-GGTG-3′), amplifying a 480 bp fragment of a Mal d 1 homologous gene (LTP-F 5′-ATGGCTTGCTCTG-CAGTGA-3′ and LTP-R 5′-TYACTTCACGGTGGCG-CAGTTG-3′), amplifying a 346 bp fragment of a Mal d 3 homologous gene and (ChiIII-Fdeg 5′-AYTGGGGH-CAAAAYGGNRRHGARG-3′ and ChiIII-R 5′-CTAGA-CATCATTCTTGATGGAG-3′), amplifying a 804 bp fragment of an apple chitinase III homologous gene in raspberry.

PCR amplifications were conducted in a total volume of 25 μL using 1 × PCR Buffer (QIAGEN), 2 mM $MgCl_2$, 0.2 mM dNTPs, 5 pmol of each Primer, 0.6 units HotStarTaq Polymerase (QIAGEN HotStarTaq™ PCR) and 4 μL of the RT reaction. PCR-cycling conditions were optimised and consisted of an initial denaturation step of 95°C for 5 min followed by 35 cycles of 60 s at 95°C, 60 s at the corresponding annealing temperature and 60 s at 72°C. A final step of 5 min at 72°C ended the cycle.

The RT-PCR products were purified using QIAquik™ PCR Purification Kit (QIAGEN) and sequenced by VBC-Biotech (http://www.vbc-biotech.com, Vienna).

2.2 Sequence data analysis

DNA sequences were analysed using Lasergene99 software (DNASTAR, Madison). Protein and DNA similarity searches were performed using BLAST program. Multiple DNA and amino acid sequence alignments were performed with Multiple sequence alignment (http://bioinfo.genopole-toulouse.prd.fr/multalin/ multalin.html).

2.3 SDS-PAGE and 2-DE

Proteins were extracted from fresh raspberries using a previously described protocol [24]. Briefly, raspberries were shock frozen, homogenised with extraction buffer (10 mM K_2HPO_4, 10 mM KH_2PO_4, 0.27 mM EDTA, 13.3 mM DIECA, 2% PVPP, pH 7) in a ratio of 1:2 w/v and extracted for 30 min by stirring at +4°C. The mixture was filtered through two layers of Miracloth (Calbiochem, USA), centrifuged at 10 000 × *g* for 30 min at +4°C and the supernatant was immediately frozen at –80°C. Phenol precipitation was used to increase the protein concentration [25]. Powdered raspberries were submitted to three washing steps with organic solvents (10% TCA/aceton, Methanol, 80% acetone) followed by an extraction with tris-buffered Phenol (pH 8, Sigma). After precipitation with a mixture containing ammonium acetate and methanol, the protein pellet was resolved in 8 M urea and the total protein content determined using the BCA Protein assay kit (Pierce, Rockford, USA), according to manufacturer's instruction.

Samples were diluted 1:2 with sample buffer containing 0.125 M Tris/HCl, pH 6.8, 20% v/v glycerol, 2% w/v SDS,

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Table 1. Clinical symptoms and sensitisation pattern of eight female patients

Patient	Age	Clinical food allergy	SPT to common inhalant allergens	Specific IgE (kUa/L)	Positive prick-to-prick test with food	Prick-to-prick test raspberry	Total IgE
1	46	OAS from stone fruits, carrot, kiwi, tree nuts, peanut	Birch	rBet v 1 22.9	Apple, peach, cherry, strawberry, kiwi, almond, potato, celery	+	88
2	34	OAS from stone fruits, hazelnut, almond	Birch, grass, *Alternaria*, dog, horse, rubber latex	Birch 15.1, latex 1.4, rHev b 6 <0.35	Apple, peach, cherry, strawberry, almond, hazelnut, bell pepper, lettuce	Nd	400
3	26	Mild OAS from apple, peach, carrot, kiwi; severe OAS from orange, asparagus, banana, melon	Ragweed, cat, house dust mite, rubber latex	Birch <0.35, rBet v 6 <0.35, latex 2.9, rHev b 6 <0.35	Peach, banana, melon, cucumber, orange, asparagus, tomato, potato	–	160
4	20	Erythema and pruritus from peach, kiwi, orange	Grass, house dust mite, *Alternaria*	Birch <0.35, grass 0.84	Nd	Nd	50
5	42	Severe OAS, urticaria, angioedema and collapse after apple and peach	Negative	rBet v 1 <0.35	Apple, peach, cherry, plum, apricot	–	220
6	30	Urticaria and oedema of the lips from tomato; pruritus, nausea and vomitus from apple and banana	Birch, grass, mugwort, ragweed, cat, dog, house dust mite	rBet v 1 9.3, rBet v 2 <0.35, rBet v 6 10.8, grass 3.1, mugwort 1.96	Apple, peach, banana, carrot, celery, tomato, bell pepper, lettuce	+	300
7	44	OAS from apple, facial angioedema from peach, anaphylaxis from apricot	Negative	Birch <0.35	Apple, peach, cherry, apricot	+	130
8	25	Periorbital edema and rhinitis from lemon and other citrus fruits	Negative	Birch <0.35, mugwort <0.35, latex <0.35	Peach, lemon, sweet lime, orange, banana, blueberry, tomato, grape, bell pepper	++	30

SPT: skin prick test, OAS: oral allergy syndrome, nd: not determined.

0.005% v/v bromphenol blue and denatured for 10 min at 90°C. Whenever reducing conditions were required, 0.05 M DTT was added to the mixture. Samples were loaded on 4–20% gradient gels (4–20% tris-glycine gel, Invitrogen, Carlsbad, USA). Electrophoresis was performed with a Novex System (Invitrogen). Precipitated raspberry proteins were separated by 2-DE as described before [20]. Samples were diluted in rehydration buffer (6 M urea, 2 M thiourea, 2% w/v CHAPS, 10 mM DTT, 2% IPG-buffer and traces of bromphenol blue) and 70 µg protein of the total protein extract or were loaded on 7 cm IPG strips, pH 3–10 or 4–7 (Amersham Biosciences, Piscataway, USA), using in-gel rehydration. Focussing was carried out on a Multiphor II System (Amersham Biosciences) at +20°C for a total of 7.2 kVh with a voltage maximum at 3500 V. Prior to the second dimension, strips were equilibrated for 12 min in equilibration solution (50 mM Tris-HCl buffer, pH 6.5 containing 6 M urea, 30% v/v glycerol, 2% w/v SDS) containing 2% DTT and subsequently for 8 min in equilibration buffer containing 2.5% iodacetamide. Small size gels were chosen for the 2-DE analyses due to the limited amounts of available patient sera.

2.4 Western blotting

Electroblotting was performed on the XCell II Blot Module (Invitrogen) using a buffer containing 50 mM $Na_2B_4O_7 \cdot 10H_2O$, 0.1% w/v SDS, 20% v/v methanol. For the localisation of Mal d 1 and Mal d 3 homologues in raspberry, polyclonal rabbit antibodies were used in Western blots as previously described [20]. Sera of mono- and polysensitised patients, who display symptoms after inhalation or consumption of pollen and fruits like apple, peach, apricot, raspberry and different vegetables were used to identify distinct IgE-reactive epitopes in raspberry extract (Table 1). For this purpose allergic individuals were clinically characterised by anamnesis, skin testing and IgE assay. Raspberry proteins were blotted onto nitrocellulose membranes (Bio-Rad, Hercules, USA) and blocked with PBS buffer containing 3% BSA and 0.1% Tween 20 for 2 h at 37°C. Membranes were first incubated over night at 4°C with patient sera diluted 1:50 v/v and subsequently with a goat anti-human IgE conjugated with horseradish peroxidase (ICL Lab, Newberg, USA) diluted 1:1000 v/v for 4 h at room temperature. IgE-reactive protein spots were visualised by ECL (Amersham Biosciences) reaction and chemilumines-

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cence using the Lumi-Imager™ apparatus (Boehringer Mannheim/Roche, Penzberg, Germany).

2.5 Inhibition assay

In order to demonstrate crossreactivity between Mal d 1 and Rub i 1, its putative homologue in raspberry, an inhibition assay was performed, assuming that only a protein with similar epitopes as Mal d 1 should be able to reduce the IgE binding. For this purpose approximately 4 µg of purified apple Mal d 1 were separated by electrophoresis and blotted on a nitrocellulose membrane. Serum of a patient allergic to Mal d 1 was diluted 1:50 v/v in PBS, containing 1% BSA and 0,1% Tween 20, and preincubated with raspberry extract (1 mL, ~1 mg/mL) for 1 h at room temperature. The blot membranes were developed by Western blotting.

2.6 Glycoproteomic analyses

Three major bands at a M_r of about 30 kDa were subjected to extensive glycoproteomic analyses using LC-ESI-Q-TOF MS and MS/MS [26]. *N*-glycans were released, isolated and analysed according to a standard method [27]. The peptides were subjected to LC-ESI-Q-TOF MS before and after digestion with peptide N/glycosidase A [28]. *De novo* determined peptide sequences were subjected to an MS-BLAST (http://dove.embl-heidelberg.de/Blast2/msblast.html) allowing the assignment of proteins by sequence similarity.

3 Results

3.1 Sequence comparison of Rub i 1, Rub i 3 and *Rubus* class III chitinase

Single strand cDNAs corresponding to Rub i 1, and Rub i 3 and class III chitinase could be amplified by PCR using primers designed on the basis of high sequence homologies to Mal d 1, Mal d 3 and apple class III chitinases [21, 29, 30] with optimised annealing temperatures (65°C for Rub i 1, 56°C for Rub i 3 and 50°C for chitinase III). Purified PCR products were sequenced and compared to *in silico* available sequence data.

The search in protein sequence databases using the BLAST and FASTA3 programs pointed to a high homology of the isolated raspberry cDNA with homologous proteins in Rosaceous and other plants. The amino acid sequence of Rub i 1 (Acc. no. DQ660361) obtained from the conversion

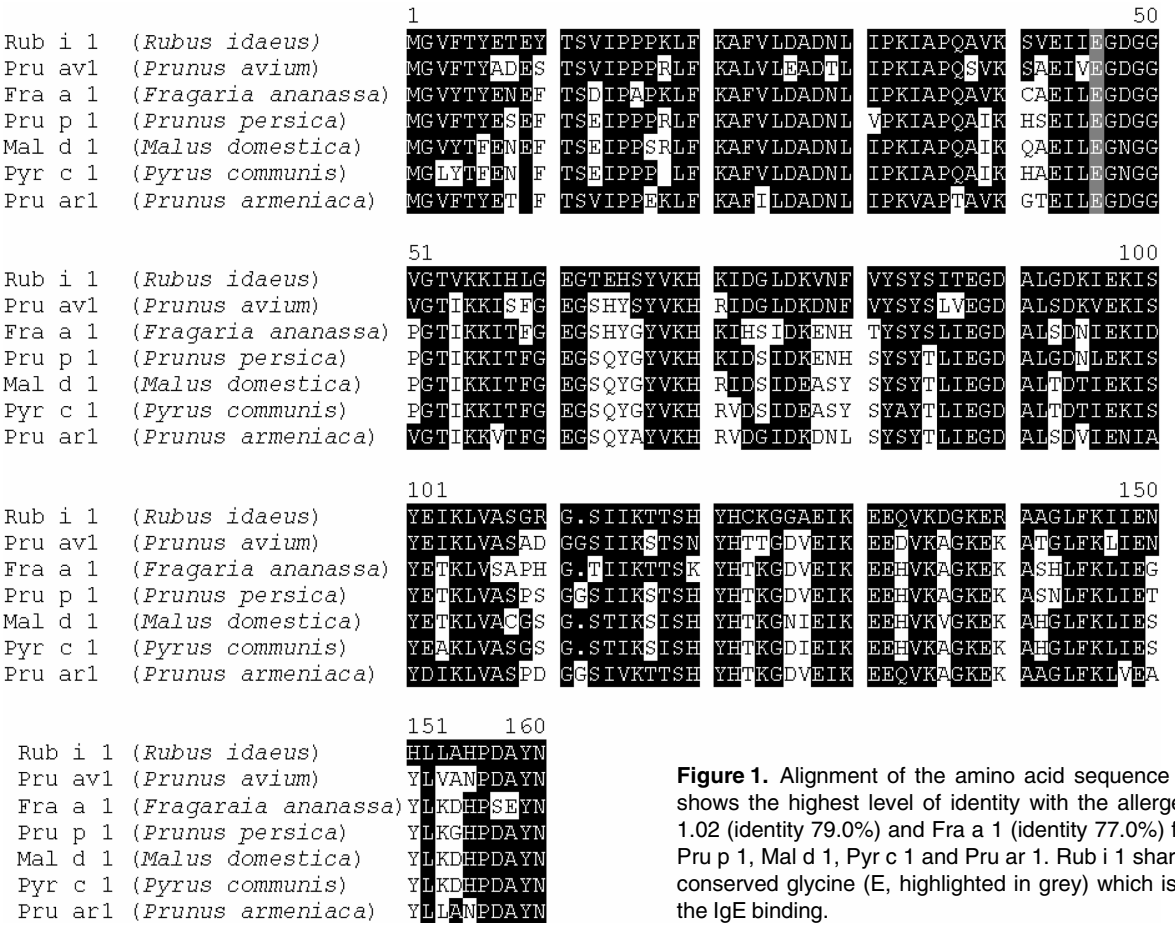

Figure 1. Alignment of the amino acid sequence of Rub i 1 shows the highest level of identity with the allergens Pru av 1.02 (identity 79.0%) and Fra a 1 (identity 77.0%) followed by Pru p 1, Mal d 1, Pyr c 1 and Pru ar 1. Rub i 1 shares also the conserved glycine (E, highlighted in grey) which is crucial for the IgE binding.

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                                1                                                  50
Rub i 3   (Rubus idaeus)        MACSAVMKLA LVALLCIVVA VPVAQAITCG QVTQNVAPCF NYVKNGGAVP
Fra a 3   (Fragaria ananassa)   MASSTAMKLS LVALLCIVVA LPIAQAITCG QVASNISPCL TYVKSGGAVP
Mal d 3   (Malus domestica)     MACSAVIKLA LVVALCMAVS V..AHAITCG QVTSSLAPCI GYVRSGGAVP
Pyr c 3   (Pyrus communis)      MASSAVIKLA LVVALCMAVS V..AHAITCS QVSANLAPCI NYVRSGGAVP
Pru av3   (Prunus avium)        MACSAMTKLA LVVALCMVVS VPIAQALTCG QVSSNLAPCI AYVRGGGAVP
Pru p 3   (Prunus persica)      MAYSAMTKLA LVVALCMVVS VPIAQAITCG QASSSLAPCI PYVRGGGAVP
Pru ar3   (Prunus armeniaca)    .......... .......... ......ITCG QVSSSLAPCI GYVRGGGAVP

                                51                                                100
Rub i 3   (Rubus idaeus)        AACCNGVRNL NSQAKTTADR QQTCNCLKNA AGSIPGLNPS LAAGLPGKCG
Fra a 3   (Fragaria ananassa)   AACCSGIRNL NGMAKTTADR QAACNCLKQA AGGIKGLNPN LAAGLPGKCG
Mal d 3   (Malus domestica)     PACCNGIRTI NGLARTTADR QTACNCLKNL AGSISGVNPN NAAGLPGKCG
Pyr c 3   (Pyrus communis)      PACCNGIKTI NGLAKTTPDR QAACNCLKNL AGSVSGVNPG NAESLPGKCG
Pru av3   (Prunus avium)        PACCNGIRNI NNLAKTTADR QTACNCLKQL SASVPGVNAN NAAALPGKCG
Pru p 3   (Prunus persica)      PACCNGIRNV NNLARTTPDR QAACNCLKQL SASVPGVNPN NAAALPGKCG
Pru ar3   (Prunus armeniaca)    PACCNGIRNV NNLARTTPDR RTACNCLKQL SGSISGVNPN NAAALPGKCG

                                101          117
Rub i 3   (Rubus idaeus)        VSVPYKISTS TNCATVK
Fra a 3   (Fragaria ananassa)   VSVPYKISTT TNCAAVK
Mal d 3   (Malus domestica)     VNVPYKISTS TNCATVK
Pyr c 3   (Pyrus communis)      VNVPYKISTS TNCATVK
Pru av3   (Prunus avium)        VNVPYKISPS TNCATVK
Pru p 3   (Prunus persica)      VSIPYKISAS TNCATVK
Pru ar3   (Prunus armeniaca)    VNIPYKISAS TNCATVK
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Figure 2. Alignment of Rub i 3 shows the highest level of identity with the allergens Fra a 3 (identity 87%) and Mal d 3 (identity 74%). Further it shares 8 cysteine-residues (highlighted in grey) with all other LTPs from *Rosaceae* like Pyr c 3, Pru av 3, Pru p 3 and Pru ar 3, which are crucial for the conformational stability of these allergens.

of the DNA sequence showed a 79.2% identity with the cherry allergen Pru av 1.02 (Acc. no. Q6QHU2), 77.1% identity with the strawberry allergen Fra a 1 (Acc. no. Q2I6V8) and 77.0% with Mal d 1.06B (Acc. no. AAS00046) from apple (Fig. 1). The amino acid sequence of Rub i 1 shares additionally a conserved glycine with these proteins, shown to be crucial for the IgE binding [31]. In the case of Rub i 3 (Acc. no. DQ660360) 87% identity could be observed with Fra a 3 from strawberry (Acc. no. Q4PLT5), 74% identity with Mal d 3 from apple (Acc. no. Q5GLH0) and 70% identity with Pru av 3 from cherry (Acc. no. Q9M5X8) (Fig. 2).

The converted partial cDNA sequence of *Rubus* class III chitinase showed the highest amino acid sequence homology with an apple class III chitinase (88.12% identity, Acc. no. Q9FUD7), 82.9% identity with a strawberry class III chitinase (Acc. no. Q9XF93) and 74.17% identity with hevamine (Acc. no. Q9FEY6), a minor latex allergen (Fig. 3).

3.2 Detection and identification of IgE-reactive raspberry proteins

Raspberry extract was separated by SDS-PAGE and transferred to nitrocellulose membranes. Protein bands were detected by Western blotting using rabbit polyclonal antibodies directed to purified Mal d 1 and Mal d 3, respectively, confirming the expression of homologous proteins in raspberry fruit (Fig. 4). Tested patient sera (35%) reacted with a protein band at 9 kDa and 60% with a protein band at 17.5 kDa (Fig. 4). Although raspberry LTP (Rub i 3) could be detected both by polyclonal and patient antiserum, it was not stained with CBB in 2-DE. Protein spots, which were detected by a pAb against Mal d 1 were identified by MS analyses as a highly abundant latex-like protein from raspberry and a cyclophilin in 2-DE (Fig. 5). Narrow range IPG-strips were used to improve the resolution of the spots reactive to Mal d 1 serum. However, the obtained MS results delivered sequence fragments of a latex-like protein from raspberry. Inhibition assay using Western blotting and serum of an apple/birch reactive patient showed that the Mal d 1 band completely disappears, when the serum is incubated with raspberry extract (Fig. 6). Additionally, the MS/MS analysis identified an IgE-reactive cyclophilin at a p*I* of 9 and M_r of 18 kDa (Fig. 7). The sequence analysis of cyclophilin peptide fragments shows a high similarity with Bet v 7, a cyclophilin from birch (*Betula verrucosa*), Gly m cyclophilin from soyabean (*Glyine maxima*) and Mal s 6 from the fungus *Malassezia sympodialis* (Fig. 8).

3.3 Characterisation of *Rubus* class III chitinase and analysis of *N*-glycans using MS

About 80% of patient sera reacted with a triple protein band at *ca*. 30 kDa (Fig. 4). These protein bands were visualised by Coomassie staining and Western blotting (Fig. 7), individually isolated, sequenced by MS/MS and identified as a class III chitinase. The raspberry extract was also separated by 2-DE and Western blotted with a reactive patient serum (Fig. 7). The triple protein band was resolved in four spots at an estimated p*I* of 8.5 (Fig 7). All four spots analysed by MS contained identical peptide fragments. LC-ESI-Q-TOF MS analysis indicated that *Rubus* class III chitinase is a gly-

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                                1                                                  50
Peptide (Rubus idaeus)                                        GG LATYWGQDGN GNEGTLR
cDNA    (Rubus idaeus)          .......... .......... ...............WGQ..N GNEGTLXQTC
Hev B   (Hevea brasiliensis)    ..MAKRTQAI LLLLLAISLI MSSSHVDGGG IAIYWGQ..N GNEGTLTQTC
Chi III (Ficus awkeotsang)      .........M ILVFLLLQAL ASIKASHAGG IAIYWGQ..N GNEGTLSQTC
Chi III (Trifolium pratense)    ..MAKNSNTF LSLIFLL.FL TFVATSHAGG IAIYWGQ..N GNEGTLSEAC
Chi III (Musa paradisiaca)      MAIRSPASLL LFAFLMLALT GRLQARRSSC IGVYWGQ..N TDEGSLADAC
Chi III (Zizyphus jojube)       ...MVPQAKL VVASLILTSA LIQTSEAVGG IATYWGQYTE TEEGSLAEAC
Chi III (Malus domestica)       MASKSTATFL ..ALLSLVTL VLALGANAGG IAIYWGQ..N GNEGTLAETC
Chi III (Pyrus communis)      .......... .. ....... .. ....... .......... ..............
Chi III (Fragaria ananassa)     MASVSAAALL ..SLATL..L LLAVGSNAGG IAIYWGQ..N GNEGTLAQTC

                                51                                                100
Peptide (Rubus idaeus)
cDNA    (Rubus idaeus)          ASGNYQFVNI AFLSSFGNGQ TPTINLAGHC DPSTNECTKL SPDIKSCQAE
Hev B   (Hevea brasiliensis)    STRKYSYVNI AFLNKFGNGQ TEQINLAGHC NPAA.GGCTI VSNGIRSCQI
Chi III (Ficus awkeotsang)      ATGKYSIVNI AFLNIFGNGQ TEQINLAGHC NPAL.KTCTV ISDGIRSCQS
Chi III (Trifolium pratense)    ATGKYTHVII AFLNKFGNGQ TEEMNLAGHC NPSVPNSCTK FSSEIKDCQS
Chi III (Musa paradisiaca)      ATGNYEYVNI ATLFKFGMGQ TEEINLAGHC DPRN.NGCAR LSSEIQSCQE
Chi III (Zizyphus jujuba)       ASNLYSYINI AYLNIFGEGR YLSLNISGHC .....SDCTF LGEEIKACQS
Chi III (Malus domestica)       ASGNYQFVNV AFLTTFGNGQ TEAINLAGHC DPTTEECTK. LSPEIKSCQA
Chi III (Pyrus communis)        .......... .......... .......... .......... ..........
Chi III (Fragaria ananassa)       SGNYQFVNI AFHSSFGNGR TPTLNLAGHC DPSSNTCTKF SSQIKSCQA.

                                101                                               150
Peptide (Rubus idaeus)              VFLSLG GASGTYSLSS AAWK
cDNA    (Rubus idaeus)          .GIKVILSIG GGAGSYSLAS PNDARQVATY LWNNFLGGQS S....SRPLG
Hev B   (Hevea brasiliensis)    QGIKVMLSLG GGIGSYTLAS QADAKNVADY LWNNFLGGKS S....SRPLG
Chi III (Ficus awkeotsang)      RGIKVMLSIG GGIGRYSLSS AMDAKNVADY LWNNFLGGKS L....SRPLG
Chi III (Trifolium peratense)   KGIIVLVSIG GGIGSYSLSS IEDARNVSTF LWNTFLGGKS S....SRPLG
Chi III (Musa paradisiaca)      RGVKVMLSIG GG.GSYGLSS TEDAKDVASY LWHSFLGGSA AR..YSRPLG
Chi III (Zizyphus jujuba)       QGVKIFLSLG GPYGDYHLTT DGDADRVAEQ LWSSFLGGSK STGVYQPLLG
Chi III (Malus domestica)       KGIKVILSIG GASGSYSLTS ADDARQVATY LWNNFLGGQS S....SRPLG
Chi III (Pyrus communis)        ...KVLLSIG GASGSYSLTS ADDARQVADY IWNNFLGGQS A....SRPLG
Chi III (Fragaria ananassa)     EGIKVILSVG GGWGQYSLAS SEDARQVGAY LWNNFLGGHS T....SRPLG

                                151                                               200
Peptide (Rubus idaeus)
cDNA    (Rubus idaeus)          DAVLDGVDFD IEGGTDQHWD DLARYLSGYS KK..G.KKVY LTAAPQCPF.
Hev B   (Hevea Brasiliensis)    DAVLDGIDFD IEHGSTLYWD DLARYLSAYS KQ..G.KKVY LTAAPQCPF.
Chi III (Ficus awkeotsang)      DAVLDGIDFD IELGSTKYWD SLARYLKGYS NLE...RPVY LTAAPQCPF.
Chi III (Trifolium pratense)    DAVLDGIDFD IELGSTQNWQ HLAKFLKEYS RY..G.KKVY LGAAPQCPL.
Chi III (Musa paradisiaca)      DAVLDGIDFN IAGGSTEHYD ELAAFLKAYN EQEAGTKKVH LSARPQCPF.
Chi III (Zizyphus jujuba)       DVELDGIDID IQIGPPEEYD VLARNLKDLT KDR..TRPFY LSAAPKCSAY
Chi III (Malus domestica)       DAVLDGIDFD IEGGTDQHWD DLARYLSGYS KR..G.KKVY LTAAPQCPF.
Chi III (Pyrus communis)        DAVLGDVDFD IEAGGGQFYD ELARSLNGHN GQA...KTVY LAAAPQCPL.
Chi III (Fragaria ananassa)     DAVLGDVDFD IEGGNDQYWD DLARYLSAHS KK..G.GKKY LTAAPQCPF.
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Figure 3.

coprotein carrying typical complex plant-type *N*-glycans with a core α1,3 fucose and a β1,2 xylose at least on one position. Both sugars are reported as immunogenic and frequently found on a variety of plant allergens [27].

4 Discussion

The current study focused on the identification and characterisation of raspberry IgE-reactive proteins using different molecular tools. Results obtained by IgE-Western blotting with polyclonal antibodies directed to the apple allergens Mal d 1 and Mal d 3 confirmed the expression of homologous proteins also in raspberry. Conversion of DNA sequences of Rub i 1 and Rub i 3 into amino acid sequences revealed a high similarity to homologous allergens in related Rosaceous species like strawberry, cherry, apple and peach. Fruits of the *Rosaceae* family are the plant foods most frequently involved in allergic reactions [6, 32–35]. A direct sensitisation by food allergens through the oral route is only possible when they are not destroyed by proteolysis in the digestive tract. This property is thought to be decisive for the potential of food allergens to induce severe systemic reactions [2]. However, specific medications in ulcer treatment can disturb the protein digestion so that lead to the development of IgE against even pepsin-labile allergens [10]. The nsLTPs from different fruits, *e.g.* Mal d 3, have been shown to possess such an extreme stability [11]. So far, birch pollen-food crossreactive IgE antibodies have mainly been implicated in oral allergy syndrome with milder symptoms. The main sources of crossreactivity, the major birch pollen allergen Bet v 1 and apple allergen Mal d 1, are extremely sensitive to pepsin digestion, explaining the restriction of symptoms to the oral cavity [6].

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```
                                 201                                                                  250
Peptide  (Rubus idaeus)                                                                     LLNSWNTWT
cDNA     (Rubus idaeus)          ..PDAWVGNA LNTGLFDYVW VQFYNNPPCQ YTSGDISNLE ...DAWKQWT
Hev B    (Hevea brasiliensis)    ..PDRYLGTA LNTGLFDYVW VQFYNNPPCQ YSSGNINNI. ..INSWNRWT
Chi III  (Ficus awkeotsang)      ..PDRFLGNA LNTGLFDYVW VQFYNNPPCQ YRSGAVDGL. ..LNSWSKWT
Chi III  (Trifolium pratense)    ..PDKFLGTA LQTGLFDFVW VQFYNNPPCQ Y.NGNITNL. ..VNSWNTWT
Chi III  (Musa paradisiaca)      ..PDYWLGNA LRTDLFDFVW VQFFNNESCH F.SQNAINL. ..ANAFNNWV
Chi III  (Zizyphus jojuba)       NDSDAYLWTA VETGLFDFVW VKFYNDTSCQ YNNDTAAGLD AFYRSWYDWT
Chi III  (Malus domestica)       ..PDAYVGNA LKTGLFDNVW VQFYNNPPCQ YASGDVTNL. ..EDAWKQWT
Chi III  (Pyrus communis)        ..PDAHLDGA IQAGLFDYVW VQFYNNPPCQ YADGNAMAL. ..LLNWSQWA
Chi III  (Fragaria ananassa)     ..PDANIGNA LKTGLFDNVW VQFYNNPPCQ YTSGNVTNL. ..EDAWKQWT

                                 251                                                                  300
Peptide  (Rubus idaeus)          TSV.TAGK   LGLPASTEA. .AGSG.YL                                   YGGV
cDNA     (Rubus idaeus)          SAI.PTHKIF LGLPAAPQA. .AGSG.FIPA ADLNSQVLPA IKNSAKYGGV
Hev B    (Hevea brasiliensis)    TSI.NAGKIF LGLPAAPEA. .AGSG.YVPP DVLISRILPE IKKSPKYGGV
Chi III  (Ficus awkeotsang)      TSI.SAGRIF LGLPAAPQA. .AGSG.YIPP NVLTSEILPA IQKSPKYGGV
Chi III  (Trifolium pratense)    RSV.PTRKIF LGLPAATAA. .AGSG.FTPA DVLTSQILPV IKKSRKYGGV
Chi III  (Musa paradisiaca)      MSI.PAQKLF LGLPAAPEA. .APTGGYIPP HDLISKVLPI LKDSDKYAGI
Chi III  (Zizyphus jojuba)       VSLAEGNKLL IGIPASNETD NSPLGGYIPS DVLNDQIVSV IMTSSKFGGV
Chi III  (Malus domestica)       SAI.PADKIF LGLPAAPQA. .AGSG.FIPA TDLSSQVLPA IKSSAKYGGV
Chi III  (Pyrus communis)        S.V.PATQVF MGLPASTDA. .AGSG.FIPA DALKSQVLPT ..........
Chi III  (Fragaria ananassa)     SAI.PAQQVF LGLPAAPEA. .AGSG.FIPA AALTTTVLPG IKTSDKYGGV

                                 301                                    340
Peptide  (Rubus idaeus)          MLWSR
cDNA     (Rubus idaeus)          MLWSKYYDDL DGYSASIKND V......... ..........
Hev B    (Hevea brasiliensis)    MLWSKFYDDK NGYSSSILDS VLFLHSEECM TVL.......
Chi III  (Ficus awkeotsang)      MLWSKYYDDK NGYSSIIFQS V......... ..........
Chi III  (Trifolium pratense)    MLWSRFFDGQ TGYSTSIIGS V......... ..........
Chi III  (Musa paradisiaca)      MLWTRYHDRN SGYSSQVKSH VCPARRFSNI LSMPVKSSK.
Chi III  (Zizyphus jojuba)       .VWNRYYDLK TNYSSSIILE YVNSGTKYLP LRTKFMYQNA
Chi III  (Malus domestica)       MLWSKYYDDP DGYSSSIKND V......... ..........
Chi III  (Pyrus communis)        .......... .......... .......... ..........
Chi III  (Fragaria ananassa)     MLWSKYYDDL YGYSSSIKNH V......... .........
```

Figure 3 (continued). Sequence alignment of the identified partial cDNA sequence and peptide fragment sequences from the *Rubus* class III chitinase. The conserved sequences are marked in black and show a high similarity to *Rosaceae* chitinases. Also the IgE reactive peptide fragments are similar to allergenic chitinase class III from jujube.

Results obtained indicate that raspberries share a similar allergen pattern with apples. Rub i 1 and Rub i 3 show a sequence identity higher than 70% with homologous allergens in other Rosaceous fruits. In fact, proteins sharing more than 70% identity are commonly considered as cross-reactive [36]. The FAO/WHO expert panel recommendations even include an identity greater than 35% over 80 or more amino acids as a guideline to suggest possible cross-reactivity (FAO Corporate document repository; http://www.fao.org/docrep/003/x7133m/x7133m03.htm). However, the expression level of Rub i 1 in the analysed samples was too low to be visualised by Coomassie staining. An IgE-reactive protein spot of 18 kDa and a p*I* 9.0 was identified by MS/MS as a cyclophilin, with high amino acid homology to the birch pollen allergen Bet v 7. Therefore, the IgE-reactivity at approximately 18 kDa is caused by at least two proteins, namely Rub i 1 and a cyclophilin. Cyclophilins have been identified to catalyse peptidyl–prolyl isomerisation, during which the peptide bond preceding proline is stabilised in the *cis*-conformation, in all organisms

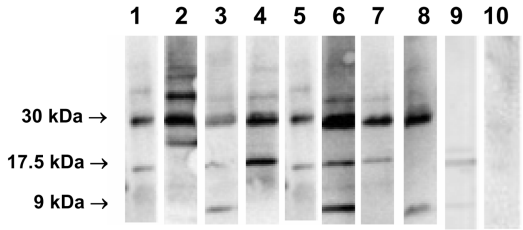

Figure 4. Eight patient sera (1 to 8) were used to screen IgE reactive proteins of raspberry by Western blotting. Polyclonal rabbit antisera raised against Mal d 1 (17.5 kDa) and Mal d 3 (9 kDa) identified the corresponding proteins in raspberry extract (9). A mixture of five non-allergenic patient sera was applied as negative control (10).

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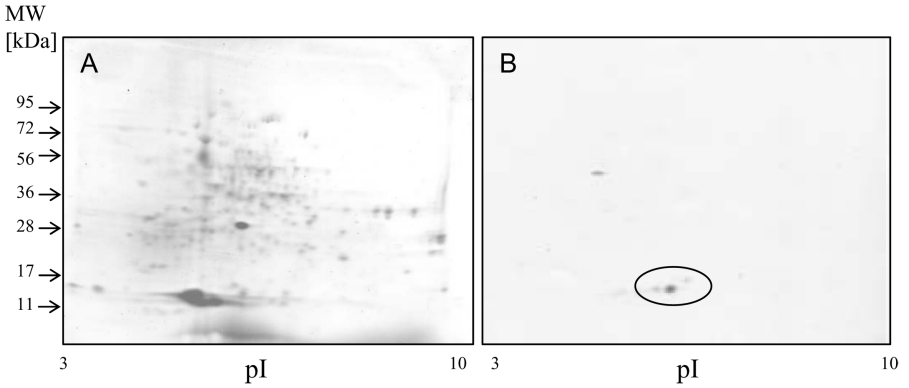

Figure 5. Raspberry extract separated in the p*I* range of 3–10 using 2DE was either stained by Coomassie blue (A) or Western blotted (B) with polyclonal antibodies against Mal d 1. Rub i 1 was reliably detected (highlighted by a black elipse).

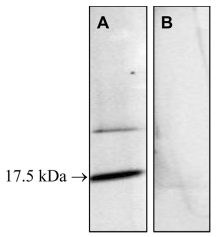

Figure 6. Inhibition assay for Mal d 1 at 17.5 kDa. Purified Mal d 1 was separated by electrophoresis and Western blotted with (A) untreated serum of an apple/birch reactive patient and (B) serum pre-incubated with raspberry extract.

studied so far [37]. Mammalian cyclophilin is a major cellular target for the immunosuppressive drug cyclosporin. Other roles for cyclophilins include chaperone and cell signalling functions [38]. So far, allergenic cyclophilins were found in fungi and plants, including Asp f 11 and Asp f 27 from *Aspergillus fumigatus* and Mal s 6 from *M. sympodialis* [37]. An IgE binding cyclophilin from carrot is not cross-reactive with the birch pollen homologue [39], a feature which still needs to be investigated for the raspberry cyclophilin.

More significant was a triple protein band of *ca*. 30 kDa reacting with 80% of the tested patient sera. The MS analysis of each single protein spot confirmed all spots as class III chitinases carrying at least one *N*-glycan with a core α1,3 fucose and a β1,2 xylose, both known to be crucial for CCDs [22]. A class III chitinase from Indian jujube, Ziz m 1, has been reported recently as allergen [40]. The protein sequence of Ziz m 1 indicates at least five possible glycosylation sites. Due to high sequence identity of class III chitinases and Hev b hevamine, a minor allergen from latex, the IgE-reactivity might be explained in the context of the latex-fruit syndrome [41]. However, reported allergies to banana and kiwi are caused by class I chitinase panallergens, which do not share any sequence similarity with class III chitinases. Class III chitinases lacking hevein domains are mainly of plant and fungal origin [40]. In the current study, the IgE-reactivity to class III chitinases was unexpected, since sera were obtained from patients with a predominant allergy to apples and raspberries, particularly to allergens like Mal d 1 and Mal d 3. Moreover the anamnesis contained only two cases of latex sensitivity, both of which were negative to Hev b 6.

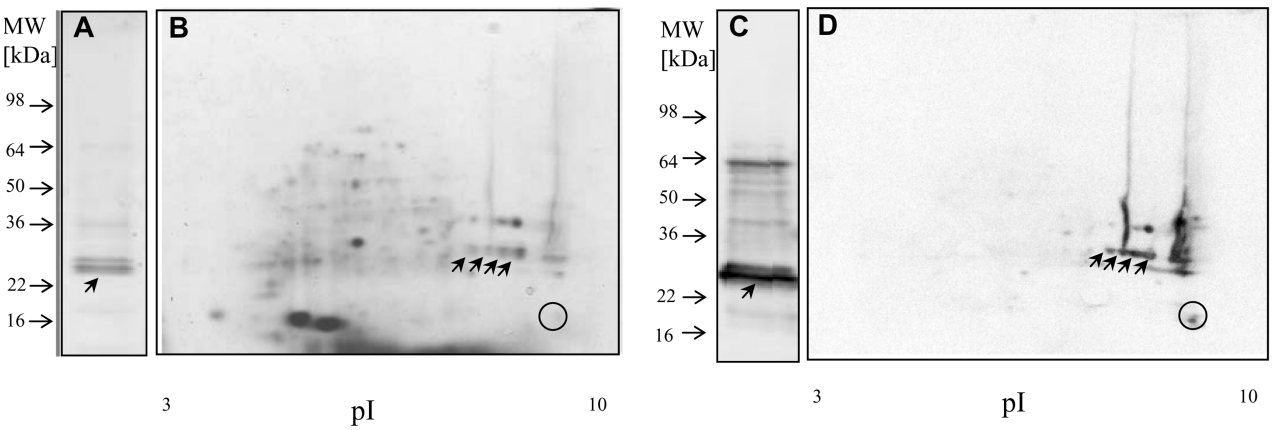

Figure 7. Separation of raspberry extract using SDS-PAGE (A,C) and 2DE (B,D). Gels were stained with Coomassie Blue (A, B) and Western blotted using a patient serum (C and D). The spots of 30 kDa and basic p*I* correspond to a class III chitinase (black arrows; figure B and D). An additional IgE-reactive spot of 18 kDa and p*I* 9.0 was identified as a cyclophilin (black circle; figure B and D).

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```
                                  1                                                  50
Rub i c (Rubus idaeus)            ......VFFD MTLGGQS.QR LVMELYADNV NK........ ..........
Bet v 7 (Betula verrucosa)        MASNPKVFFD MEVGGQPVGR IVMELYADTT PRTAENFRAL CTGEKGNGRS
Gly m c (Glycine maxima)          .MPNPKVFFD MTIGGQSAGR IVMELYADVT PRTAENFRAL CTGEKGVGRS
Mal s 6 (Malassezia sympodialis)...MSNVFFD ITKNGAPLGT IKFKLFDDVV PKTAANFRAL CTGEKGFG..
                                  51                                                 100
Rub i c (Rubus idaeus)            .......... .......... .......... .......... FADENFVK..
Bet v 7 (Betula verrucosa)        GKPLHYKKSS FHRVIPGFMC QGGDFTAGNG TGGESIYGAK FADENFIKKH
Gly m c (Glycine maxima)          GKPLHYKGSS FHRVIPSFMC QGGDFTAGNG TGGESIYGAK FADENFVKKH
Mal s 6 (Malassezia sympodialis)..... YAGSH FHRVIPDFML QGGDFTAGNG TGGKSIYGAK FADENFQLKH
                                  101                                                150
Rub i c (Rubus idaeus)            .......... .......... .......... .......... ..........
Bet v 7 (Betula verrucosa)        TGPGILSMAN AGPGTNGSQF FICTAKTEWL DGKHVVFGQV VEGLDIVKAI
Gly m c (Glycine maxima)          TGPGILSMAN AGPGTNGSQF FICTEKTEWL DGKHVVFGQV IEGLNVVKDI
Mal s 6 (Malassezia sympodialis)NKPGLLSMAN AGPNTNGSQF FITTVVTSWL DGKHVVFGEV IDGMNVVKAI

                                  151                 173
Rub i c (Rubus idaeus)            .......... .......... ...
Bet v 7 (Betula verrucosa)        EKVGSSSGRT SKPVVVADCG QLS
Gly m c (Glycine maxima)          EKVGSGSGRT SKPVVTANCG QPS
Mal s 6 (Malassezia sympodialis)EAEGSGSGKP RSRIEIAKCG VC.
```

Figure 8. The alignment of raspberry cyclophilin peptide fragments reveals a high similarity with Bet v 7, a minor birch pollen allergen, Gly m c, a cyclophilin from *Glycine maxima* and Mal s 6, an allergen from the fungus *Malassezia sympodialis.*

The present study answers two main questions: first, the raspberry fruit shares at least two IgE-reactive proteins with other Rosaceous fruits, namely Rub i 1, a Mal d 1 homologous protein and Rub i 3, a Mal d 3 homologous nsLTP. Second, proteomic investigations indicate that the IgE-reactivity of raspberry proteins is not only induced by known PR genes, but also by additional gene families and panallergenic proteins like cyclophilins and class III chitinases. However, more clinical investigations are necessary to understand the impact of crossreactivity of class III chitinase and latex hevamine or raspberry cyclophilin and birch Bet v 7.

The authors have declared no conflict of interest.

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