

Original article

Napins, 2S albumins, are major allergens in oilseed rape and turnip rape

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Background: Children with IgE-mediated allergy to foods frequently react to seeds of oilseed rape (*Brassica napus* ssp. *oleifera*) and turnip rape (*Brassica rapa* ssp. *oleifera*) in skin prick tests (SPTs). Sensitization pathways are not known. **Objective:** We identified possible major allergens in oilseed rape and turnip rape using sera from 72 atopic children (mean age, 3.3 years) with positive SPT responses to oilseed rape and turnip rape.

Methods: Allergens from oilseed rape and turnip rape seed extracts were purified by using gel filtration and cation exchange chromatography and characterized by means of reversed-phase chromatography, N-terminal amino acid sequencing, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. IgE binding of sera from 72 children with positive SPT reactions to oilseed rape and turnip rape and 72 age- and sex-matched atopic control subjects with negative SPT responses were analyzed by means of IgE ELISA and immunoblotting. *In vivo* reactivity of the purified allergens was tested with SPTs in 6 children.

Results: In IgE immunoblotting and IgE ELISA major reactivity was to a group of homologous, approximately 9.5- to 14.5-kD proteins. These allergens were identified as 2S albumins, also known as napins, by means of N-terminal amino acid sequencing. In ELISA approximately 80% of the patients had IgE to purified napins from both plants. In SPTs purified napins caused positive reactions in all 6 children tested.

Conclusions: This study shows that 2S albumins in oilseed rape and turnip rape are new potential food allergens. Further studies are needed to clarify the routes of exposure and mechanisms of sensitization. (J Allergy Clin Immunol 2005;115:1009-1016.)

Key words: Food allergy, Brassicaceae, oilseed rape, turnip rape, 2S albumin, napin

Abbreviations used

MALDI-TOF: Matrix-assisted laser desorption/ionization time-of-flight
 PVDF: Polyvinylidene difluoride
 RP: Reversed phase
 SPT: Skin prick test

The Joint Food and Agriculture Organization of the United Nations/World Health Organization Technical Consultation on Food Allergies concluded in 1995 that the major contributors to IgE-mediated food allergy worldwide are milk, egg, fish, crustacean, wheat, peanut, tree nut, and soy.¹ However, regional differences and alterations in food consumption have been suggested to affect the prevalence of certain allergies.² In addition to these frequently listed allergens, for example, potato seems to be an important allergen in Finnish, mustard in French, and sesame seed in Israeli children.²⁻⁴

We recently found that many infants and young children suspected of having food allergy frequently had positive skin prick test (SPT) reactions to seeds of oilseed rape (*Brassica napus* ssp. *oleifera*) and turnip rape (*Brassica rapa* ssp. *oleifera*, formerly *Brassica campestris*).⁵ These closely related plants are used in vegetable oil production, and their cultivation has increased sharply in the last 2 decades. Oilseed rape ranks as the most commonly grown oilseed crop in Europe,⁶ whereas turnip rape is also commonly used in Finnish agriculture and food industry.⁷

Previous studies concerning possible allergenic properties of oilseed rape and turnip rape have focused on the pollens of these plants. Three allergens or allergen groups from oilseed rape pollen have been described, but their clinical importance is controversial because very little airborne pollen is transported over longer distances.⁸⁻¹³ Data on turnip rape pollen are more limited.^{14,15} In addition, a few case reports of occupational allergies to oilseed rape dust have been published.¹⁶⁻¹⁸ In one of these, a hydrophilic 2S albumin was shown to be allergenic for one patient.¹⁶ Allergens belonging to 2S albumin family

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have previously been characterized, for example, from white mustard (*Sinapis alba*, Sin a 1) and oriental mustard (*Brassica juncea*, Bra j 1).^{19,20} Interestingly, in France mustard allergy is now ranked in the fourth place in children's food allergies.²¹

We report here identification and characterization of 2S albumins from 2 botanically related oil plants, oilseed rape and turnip rape, and suggest that they represent previously unnoticed food allergens in children.*

METHODS

Patient and control sera

Sera were obtained from 72 children with atopic dermatitis (mean age, 3.3 years; age range, 0.5–15.4 years; 47 male subjects; mean total IgE, 915 kU/L [SD, 1551]) who had clearly positive reactions (size of the wheal, ≥ 5 mm; median of 7 mm to oilseed rape and 6 mm to turnip rape) to seeds of oilseed rape, turnip rape, or both on SPTs. Twenty-five of a subset of 28 subjects were allergic to seeds of turnip rape, as confirmed in labial and open food challenges.⁵ Thirty of the 72 patients had asthma. Fifty-two (72%) of them were allergic to cow's milk, and 52 (72%) were allergic to wheat, as confirmed by means of open food challenges. On SPTs, 66 (92%) of them reacted to egg, and 69 (96%) reacted to mustard. Control sera were obtained from 72 age- and sex-matched atopic children with atopic dermatitis (mean age, 3.3 years; age range, 0.4–16.6 years; 47 male subjects; mean total IgE, 418 kU/L [SD, 1107]) who had negative SPT reactions (size of the wheal, ≤ 2 mm) to oilseed rape and turnip rape. Twenty-five (35%) of them were allergic to cow's milk and 24 (33%) were allergic to wheat on open food challenges. On SPTs, 28 (39%) reacted to egg. The ethical committee of Tampere University Hospital approved the study, and informed written consent was obtained from the parents.

Extraction and purification of seed proteins

Seeds of oilseed rape (*Brassica napus* ssp. *oleifera* cv Hyola 38) and turnip rape (*Brassica rapa* ssp. *oleifera* cv Kultra) obtained from Mildola oy (Kirkkonummi, Finland) were ground and extracted with PBS (10 mM sodium phosphate and 0.15 M NaCl, pH 7.4) overnight at room temperature. The samples were centrifuged (4000g for 30 minutes), and supernatant was collected and filtered (0.4 μ m, Millex-HV; Millipore, Bedford, Mass).

For protein purification, the clarified seed extracts were subjected to gel filtration and cation exchange chromatography. Gel filtration was performed on a Superdex 75 HR 16/60 column (Amersham Biosciences, Uppsala, Sweden) in PBS with an ÄKTA purifier instrument (Amersham Biosciences). The flow rate was 1.5 mL/min, and the chromatography was monitored at 214 and 280 nm. Cation exchange chromatography was performed on a MonoS column (Tricorn 4.6/100 PE, Amersham Biosciences) equilibrated with 25 mM glycine, pH 9.0, with the ÄKTA purifier instrument. Elution was performed with a linear gradient of NaCl (0–1 mol/L in 40 minutes) in the equilibration buffer. The flow rate was 1 mL/min, and the chromatography was monitored at 214 and 280 nm. Buffer changes were performed on a Bio-Gel P6 desalting gel (Bio-Rad Laboratories, Hercules, Calif) column (1.0 $\times$ 11 cm) with the ÄKTA purifier instrument. The flow rate was 0.5 mL/min, and the chromatography was monitored at 214 and 280 nm.

Reversed phase (RP) chromatography was performed on a 0.1 $\times$ 15 cm Vydac column (C8, 5 μ m, 300 Å; LC-packings, Amsterdam, The Netherlands) with a SMART chromatography system (Amersham Biosciences). Elution was performed by using a linear gradient of acetonitrile (0% to 100% in 60 minutes) in 0.1% trifluoroacetic acid. The flow rate was 50 μ L/min, and the chromatography was monitored at 214 and 280 nm.

Electrophoresis and immunoblotting

Chromatography fractions from oilseed rape and turnip rape were analyzed by means of electrophoresis and immunoblotting. Proteins were separated by using SDS-PAGE in 18% or 20% gels according to the method of Laemmli²² under reducing conditions. For immunodetection, proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon Transfer Membranes, Millipore) and treated with a pool of sera (diluted 1:20) from 8 patients with positive specific IgE results. After 2 hours of incubation, biotinylated anti-human IgE (diluted 1:1000, Vector Laboratories, Inc, Burlingame, Calif) was added, followed by streptavidin-conjugated alkaline phosphatase (diluted 1:24000; Zymed Laboratories, Inc, San Francisco, Calif) and substrate (Alkaline phosphatase conjugate substrate kit, Bio-Rad Laboratories).

IgE ELISA

The microtiter plates were coated with selected protein fractions (protein concentration of 1 μ g/mL, 100 μ L per well) and postcoated with 1% human serum albumin. Sera from 72 children and 72 atopic control subjects (diluted 1:10) were added (100 μ L per well), and the plates were incubated for 2 hours. Biotinylated anti-human IgE (diluted 1:1000, Vector) was added, followed by streptavidin-conjugated alkaline phosphatase (diluted 1:3000, Bio-Rad) and substrate (pNPP; ICN Biomedicals Inc, Aurora, Ohio). The color reaction was measured at 405 nm with a Multiskan Ascent-ELISA reader (Thermo Labsystems, Vantaa, Finland). The mean value of control sera plus 3 SDs was used as the cut-off limit for a positive result.

Mass spectrometry

Mass spectrometric analyses were performed with an Ultraflex matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)/TOF mass spectrometer (Bruker-Daltonics, Bremen, Germany) equipped with a nitrogen laser operating at 337 nm. Protein samples from RP chromatographic fractions were analyzed in the linear positive ion delayed extraction mode by using α -cyano-4-hydroxycinnamic acid as a matrix (saturated solution in 60% acetonitrile and 0.1% trifluoroacetic acid). Samples were prepared by mixing 0.5 μ L of sample with 0.5 μ L of matrix on the target plate and drying under a stream of warm air. Protein standard mixtures I or II (Bruker-Daltonics) were used for calibration.

Sequence analysis

N-terminal sequencing was performed by using Edman degradation either from proteins electroblotted on PVDF membrane²³ or from RP chromatographic fractions with a Procise 494A Sequencer (Perkin Elmer Applied Biosystems Division, Foster City, Calif).

SPTs with crude seeds and purified proteins

SPTs were performed on the volar arms of the children with crushed seeds of oilseed rape and turnip rape moistened with physiologic saline by using a commercial 1-peak lancet (ALK-Abelló A/S, Hørsholm, Denmark) and the prick-prick method. Purified proteins diluted in PBS were tested in concentrations ranging from 10 to 100 μ g/mL. Histamine dihydrochloride (10 mg/mL, ALK-Abelló A/S) was used as a positive control, and Solu-Prick SQ (ALK-Abelló A/S) was used as a negative control. The mean of

*The present data concerning 2S albumins as allergens in seeds of oilseed rape and turnip rape have been accepted by the IUIS Allergen Nomenclature Sub-Committee, and the allergens are named Bra n 1 and Bra r 1 (new entry), respectively.

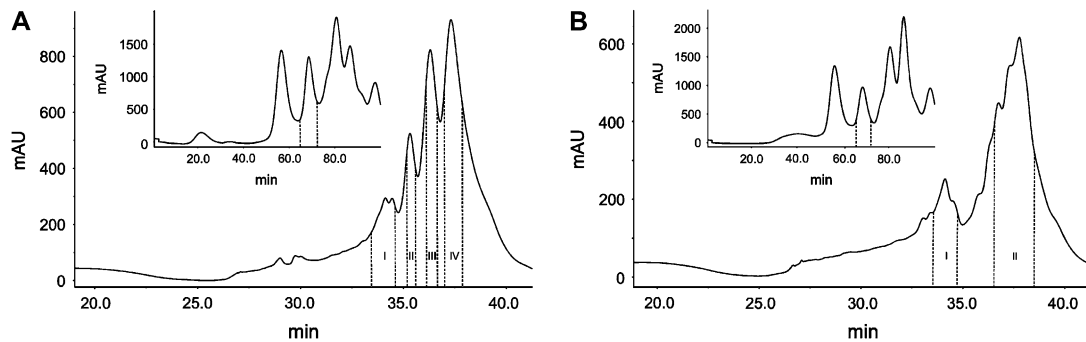


FIG 1. Purification of immunoreactive proteins from oilseed rape (**A**) and turnip rape (**B**). Marked fractions from gel filtration (*smaller picture*) of oilseed rape and turnip rape extracts were further subjected to cation exchange chromatography (*larger picture*). Four fractions from oilseed rape (*I-IV*) and 2 fractions from turnip rape (*I* and *II*) were selected for further analyses. Absorbance was measured at 280 nm.

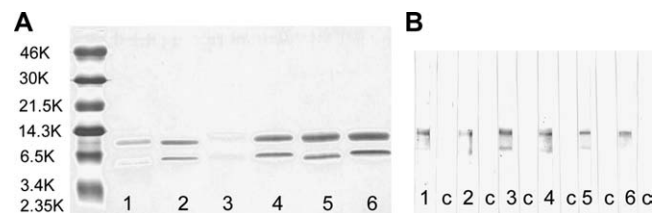


FIG 2. SDS-PAGE and Coomassie blue staining (**A**) of cation exchange and RP chromatographic purified fractions of oilseed rape and turnip rape (loaded 2 μ g per lane; lane 3, 1.2 μ g) and IgE immunoblot analysis (**B**) to cation exchange fractions with pool of sera from 8 patients. C, Atopic control; lanes 1 through 4, oilseed rape fractions I through IV, respectively; lanes 5 and 6, turnip rape fractions I and II, respectively.

2 perpendicular diameters of the wheal was measured at 15 minutes, and a wheal of 5 mm or larger was considered positive.

The statistics were calculated with the SPSS 11.5 program (SPSS Inc, Chicago, Ill) by using the Mann-Whitney *U* test or the Kruskal-Wallis test, and post-hoc testing of several univariate comparisons was performed with the Conover-Inman procedure.

RESULTS

Purification and characterization of allergens

When seed extracts from oilseed and turnip rape were subjected to SDS-PAGE and IgE immunoblotting with individual patient sera, major reactivity was in the protein area between 6 and 14 kd. To purify the major immunoreactive proteins, the seed extracts were first subjected to gel filtration, and then the immunoreactive fractions were further subjected to cation exchange chromatography (Fig 1). After cation exchange chromatography, 4 immunoreactive fractions from oilseed rape and 2 from turnip rape were selected for further analyses. SDS-PAGE and immunoblot analysis of these fractions under reduced conditions resulted in all very similar patterns, with 2 major protein bands of sizes around 4 and 9 kd (Fig 2). For identification, the proteins from cation exchange chromatography fractions were, after SDS-PAGE, electroblotted onto a PVDF membrane and subjected to N-terminal sequence analysis. The proteins from fraction 4 were first analyzed, and the upper band resulted in an N-terminal sequence of

TABLE I. N-terminal sequences determined for the 2 major protein bands in IgE-binding cation exchange fractions from oilseed rape, turnip rape, and white mustard

	N-terminal sequence	
	Small subunit	Large subunit
Oilseed rape 1	IPKCRKEFQQAQ	PQGPQQRPLLQ
Oilseed rape 2	PAGPFRIPKCR	PQGPQQRPLLQ
Oilseed rape 3	PAGPFRIPKC	PQGPQQRPLL
Oilseed rape 4	PAGPFRIPKCR	PQGPQQRPLLQ
Turnip rape 1	IPKCRKEFQQAQ	PQGPQQRPLLQ
Turnip rape 2	PAGPFRIPKCR	PQGPQQRPLLQ
Mustard 1	IPKCRKEFQQAQHLR	PQGPQQRPLLQQCC
Mustard 2	IPKCRKEFQQAQHLR	PQGPQQRPLLQQCC
Mustard 3	PAGPFGIPKCRKEFQ	PQGPQQRPLLQQCC
Mustard 4	PAGPFGIPKCRKEFQ	PQGPQQRPLLQQCC
Mustard 5	PAGPFRIPKCRKEFQ	PQGPQQRPLLQQCC

Underlined residues represent the position of a variable amino acid.

PQGPQQRPLLQ... and the lower band resulted in a sequence of PAGPFRIPKCR... (Table I). In database searches the determined sequences resulted in both a 100% match to the oilseed rape (P17333) and white mustard (P15322) 2S albumin precursors, corresponding to the large chain and small chain of the mature proteins, respectively. The N-terminal sequences determined by us correspond fully to those presented for the mature protein

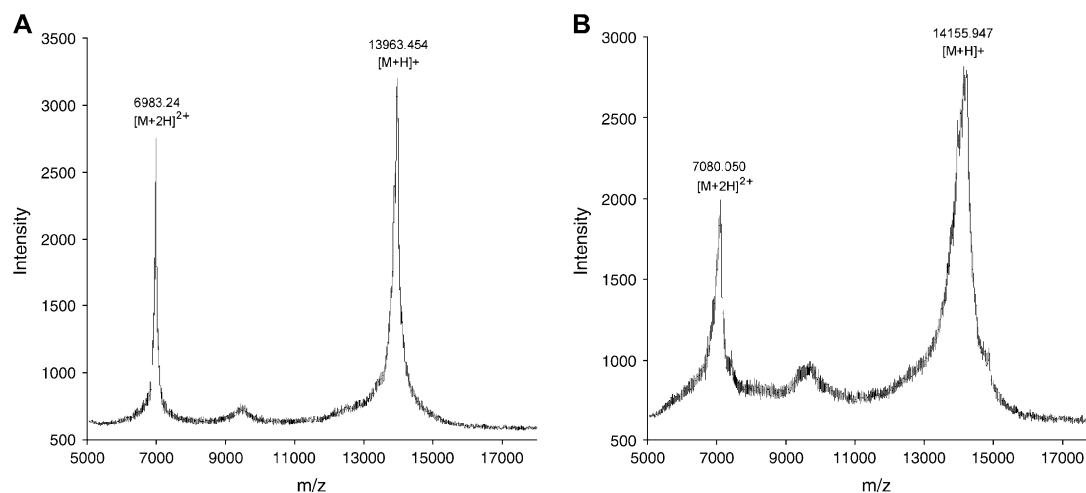


FIG 3. MALDI-TOF mass spectrum of purified napins from oilseed rape fraction II (**A**) and turnip rape fraction II (**B**). $[M+2H]^{2+}$, Doubly charged forms of the proteins.

in P15322 but indicate precursor processing 1 residue (small chain) and 3 residues (large chain) N-terminally to that suggested in P17333. N-terminal sequence analysis of the 2 major proteins from the other cation exchange fractions showed identical N-termini (Table I) to the previously analyzed ones, with one exception. The small subunit from cation exchange fraction I from both plants resulted in an N-terminal sequence of IPKCRKEFQQAQ... corresponding either to an N-terminal truncation of 6 amino acid residues from the mature protein or an altered cleavage of the precursor protein.

For comparison, we purified also the 2S albumin from white mustard (*Sinapis alba*; Paulig Oy, Helsinki, Finland) with the same methods as above. After cation exchange chromatography, 5 immunoreactive fractions with comparable elution properties and subunit composition to the oilseed rape and turnip rape 2S albumins were obtained (data not shown). Both subunits from these 5 cation exchange fractions were subjected to sequence analysis, with results as shown in Table I. In general, all determined N-terminal sequences of the 2S albumin large subunits from oilseed rape, turnip rape, and mustard were identical. In the small subunits a 1-amino-acid difference could be detected among the analyzed residues. The truncated mustard 2S albumin small subunit eluted in cation exchange chromatography as 2 peaks, whereas that from oilseed rape and turnip rape eluted as 1 peak. MALDI-TOF mass spectrometric analysis of the oilseed rape and turnip rape napins from different cation exchange fractions resulted in varying molecular masses between 9.5 and 14.5 kd, which is in accordance with expected molecular masses of napins consisting of 1 small and 1 large subunit. The mass spectra of proteins from cation exchange fractions II from oilseed rape and turnip rape are shown in Fig 3 as an example. An example of RP chromatographic run to obtain the samples for MALDI-TOF analyses is shown in Fig E1 in the Online Repository at www.jacionline.org.

IgE ELISA

ELISA assays were performed with fractions from cation exchange chromatography. Against the purified fractions from oilseed rape (I-IV) and turnip rape (I and II), approximately 80% (oilseed rape: weighted mean, 80.2%; range, 50.0% to 89.7%; turnip rape: weighted mean, 79.9%; range, 50.0% to 89.7%) of the 72 patients had positive results (ie, exceeding the mean + 3 SD of 72 atopic control children). By using age group-based cut-off levels for positive results, 5 of the control subjects showed weak responses to 1 or 2 of the purified fractions from oilseed rape and turnip rape. Patient responses as a group differed always from control responses ($P < .001$). Reactivity against different fractions from oilseed rape (I-IV) and turnip rape (I and II) was almost identical. Of the different age groups, 2- to 4-year-old patients had higher responses than 0- to 1-year-old patients ($P = .013$ for oilseed rape and $P = .012$ for turnip rape), but no other differences existed. Because the results against all fractions were almost identical, results of only one fraction from oilseed rape (fraction II) and one from turnip rape (fraction II) are presented in Fig 4.

SPT reactivity of purified napin proteins

SPTs were performed in 6 children with 4 purified fractions of oilseed rape and 2 of turnip rape. In addition to seeds, every child had positive SPT reactions to all 6 purified fractions (Table II).

DISCUSSION

Oilseed rape and turnip rape have not been considered to be potent pollen or food allergens.¹³ However, our recent SPT screening study comprising 1887 infants and young children remitted for suspected food allergy showed that as many as 11% of them reacted to oilseed rape, turnip rape, or both (size of the wheal, ≥ 5 mm).⁵

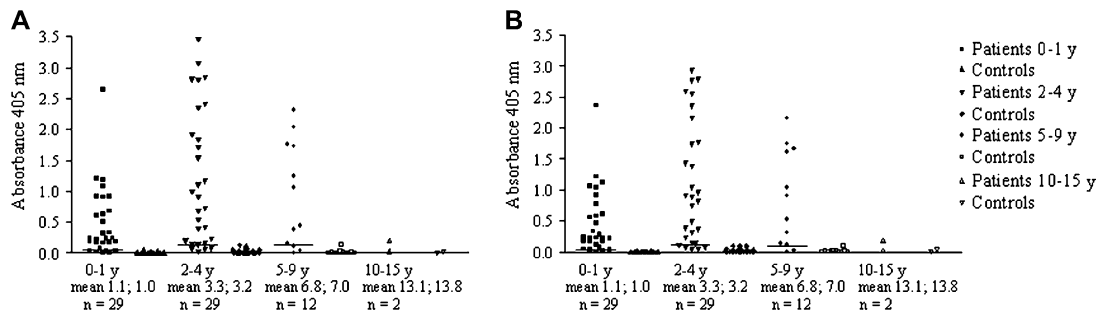


FIG 4. IgE binding to purified oilseed rape fraction II (A) and turnip rape fraction II (B) proteins of sera from sensitized children (n = 72) and atopic control subjects (n = 72) in different age groups by means of ELISA. Cut-off level for a positive response (mean OD + 3 SD of control subjects) is indicated by a horizontal line.

TABLE II. SPT reactions to cation exchange fractionated oilseed rape and turnip rape proteins in 6 children with positive reactions to seeds

Child no.	Histamine 10 mg/mL	Oilseed rape					Turnip rape		
		Fractions				Seeds	Fractions		Seeds
		I	II	III	IV		I	II	
1	3	6/50*	6/50	6/50	4/50	5	4/50	4/50	7
2	5	5/50	4/50	4/50	4/50	7	5/50	7/100	5
3	4	7/100	6/100	4/100	5/100	4	4/100	3/100	6
4	5	4/50	5/50	5/50	6/50	7	4/50	7/50	7
5	5	6/10	6/10	7/10	7/10	15	7/10	8/10	7
6	5	7/10	7/10	7/10	7/10	5	7/10	5/10	5

*Wheal diameter in millimeters/concentration in micrograms per milliliter.

In labial and oral challenges, 25 of a subset of 28 subjects were allergic to seeds of turnip rape.⁵ Here we identified and characterized possible major allergens from seeds of oilseed rape and turnip rape.

Major allergenic proteins from oilseed rape and turnip rape were extracted and purified by means of gel filtration and cation exchange chromatography, which should preserve the proteins in their native forms. Proteins in all obtained cation exchange chromatography fractions (4 from oilseed rape and 2 from turnip rape) showed similar immunoreactivity; IgE binding to purified proteins was highly interrelated between these 2 oil plants (Pearson correlation coefficient between oilseed rape II and turnip rape II fractions = 0.996) and between different fractions. This seems understandable because according to N-terminal sequence analysis, the fractions consist of a group of napins consisting of 1 small and 1 large subunit with almost identical N-termini. How these napins with different elution properties in cation exchange chromatography and with varying molecular masses differ from each other remains at present unclear. In IgE ELISA approximately 80% of the 72 patients had positive reactions to all of the purified fractions from both plants, whereas 20% had negative reactions, although they all had positive reactions on SPTs to ground seeds of oilseed rape and turnip rape. This leaves space to presently unidentified allergens.

The identified allergens, napins, are 2S albumins that are water-soluble, small-sized storage proteins synthesized during seed maturation.^{24,25} *In vitro* bioactivities, like enzyme inhibition and antifungal activity of 2S albumins, have been reported, but the *in vivo* significance of these activities is unknown.^{26,27} 2S albumins have been shown to represent allergens, for example, in sunflower seed, white mustard, oriental mustard, sesame seed, brazil nut, and castor bean.^{19,20,28-31}

At the moment, the route of sensitization of oilseed rape or turnip rape allergy is unclear. Some of the youngest patients were exclusively breast-fed. Lactating mothers and children did not consume seeds of these plants in their diet. Mixed oilseed rape and turnip rape oil is the most widely used vegetable oil in the Finnish diet,⁷ and it is also used as an ingredient in margarine. According to food labels, they are currently added also to several baby foods. In general, reactions to vegetable oils are believed to be rare and restricted to cold-pressed oils, and accordingly, rapeseed oil is not thought to be allergenic.^{32,33} However, allergic reactions to both cold-pressed and refined oils, such as peanut and sesame oil, have been reported.^{34,35} For the time being, there is no evidence to show that the oil of oilseed rape and turnip rape would cause or worsen the symptoms in our patients.⁵

Seed proteins of oilseed rape, turnip rape, and mustard, all members of the *Brassicaceae* family, have revealed

high sequence homology (93.8% identity between oilseed rape napin P17333 and white mustard 2S albumin in UniProt database), implying that IgE cross-reactions are conceivable. We performed pilot experiments using IgE ELISA inhibition (data not shown). Using purified napins of all 3 seeds, we noted considerable individual variation. The binding of a patient's IgE to oilseed rape or turnip rape napin was effectively, although to a varying degree, inhibited also by mustard napin. Extended studies are in progress to clarify these cross-reactions. Increasing prevalence of mustard allergy in France is explained partly by more attentive clinical research but also by increased consumption.² In our study, however, the consumption of mustard among the atopic children or their lactating mothers was considered to be minimal, if not negligible. Therefore the data accumulated supports the contention that mustard is not the primary allergen in our patients.

Little is known about cross-reactions between *Brassicaceae* oil plants and other plants with 2S albumins,³⁶ such as sunflower seed, sesame seed, and brazil nut. According to N-terminal amino acid sequences, the homology between oil plants within the *Brassicaceae* family is high, whereas it is lower with other plants with 2S albumins mentioned above. The consumption of different kind of seeds has increased in recent years because of increased use of ethnic dishes and bakery products, but they generally still make up only a minimal proportion of Finnish diet. From other *Brassicaceae* plants, such as broccoli, cauliflower, cabbage, and turnip, seeds are not eaten. To the best of our knowledge, no studies exist about 2S albumins as allergens from stipes, leaves, inflorescences, or tuberous roots.

Seed proteins of the *Brassicaceae* family have a nutritionally high biologic value, and therefore better use prospects of seeds and oil extraction residues have been considered for human nutrition.³⁷⁻³⁹ Given that 2S albumins are potent allergens, this subject needs thorough investigation before new products are introduced into the market. 2S albumins have been reported to be very resistant to pepsin digestion and denaturation caused by heat and low pH.⁴⁰ Thus it appears possible that these allergens remain stable in food processing.

In conclusion, we show that 2S albumins, or napins, in oilseed rape and turnip rape are new potential food allergens. Studies are needed to clarify the routes of exposure, mechanisms of sensitization, and clinical importance of this new phenomenon. Also, the possibility of cross-reactions deserves extended study.

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