

ORIGINAL ARTICLE Allergens

Molecular characterization of wheat allergens specifically recognized by patients suffering from wheat-induced respiratory allergy

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Summary

Background Wheat (*Triticum aestivum*) is an important allergen source responsible for various clinical manifestations of allergy (i.e. food allergy, pollen allergy, respiratory allergy to flour-Baker's asthma).

Objective The objective of this study was the molecular and immunological characterization of new recombinant wheat allergens and to evaluate their usefulness for the diagnosis of allergy to wheat.

Methods A *T. aestivum* cDNA library was constructed and screened with serum IgE from patients suffering from wheat allergy to identify cDNAs coding for new wheat allergens. The allergen-encoding cDNAs were expressed in *Escherichia coli* and purified to homogeneity. IgE reactivity of recombinant proteins was analysed in RAST-based, non-denaturing dot blot experiments and by ELISA with sera from wheat allergic patients and their allergenic activity was assessed in basophil degranulation experiments.

Results We report the molecular characterization, recombinant expression and purification of five wheat allergens, a thioredoxin h isoform, glutathione transferase, 1-Cys-peroxiredoxin, profilin and dehydrin. Homologous proteins were identified by sequence comparisons in various plants. 1-Cys-peroxiredoxin appeared to be the most relevant of the newly identified wheat allergens according to prevalence of IgE recognition and results from basophil degranulation experiments. It showed IgE cross-reactivity with seed proteins from barley, rye, rice, maize, soy, oat and spelt. 1-Cys-peroxiredoxin, glutathione transferase and dehydrin were mainly recognized by patients with baker's asthma but not wheat-induced food allergy.

Conclusion and Clinical Relevance The characterized recombinant wheat allergens may be useful for the development of serological tests which allow the discrimination of different clinical manifestations of wheat allergy.

Keywords Baker's asthma, recombinant allergens, *Triticum aestivum*

Abbreviations HSA, human serum albumin; Phl p, *Phleum pratense*; DBPCFC, double-blind placebo-controlled food challenge; RBL, rat basophil leukaemia; LTP, lipid-transfer protein; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonylfluoride; EDTA, ethylenediaminetetraacetic acid.

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Introduction

Wheat (*Triticum aestivum*) is a potent allergen source which causes several distinct clinical manifestations

of IgE-mediated allergy [1]. These manifestations include wheat food allergy, respiratory allergy to wheat pollen and sensitization to inhaled wheat flour as a major cause for occupational sensitization among bakers and persons processing wheat flour [2–4].

The diagnosis of the various manifestations of wheat allergy is based on anamnesis/case history, the demonstration of allergen-specific IgE antibodies in serum and provocation testing with wheat allergen extracts. The detection of wheat allergen-specific IgE antibodies is not only important for the demonstration of the IgE-mediated pathogenesis. It has also been shown that measurement of flour-specific IgE and skin prick testing may predict nasal and bronchial challenge test results in the case of wheat-induced respiratory allergy, mainly in baker's asthma [5].

In the last years many important wheat allergens have been characterized by biochemical, immunological and molecular biological methods. Interestingly, IgE-recognition of certain wheat allergens seems to be associated with defined clinical manifestations of wheat allergy. For example, omega-5-gliadin, Tri a 19, has been described as an allergen associated with wheat-dependent, exercise-induced anaphylaxis and wheat food allergy [6–9]. Alpha amylase inhibitors, wheat gliadins, lipid transfer protein and serine proteinase inhibitor have been described as allergens which may be recognized specifically by patients suffering from respiratory allergy to wheat, in particular baker's asthma [10–14]. Thioredoxins [15] and profilins [16] could be identified as allergens involved in respiratory allergy to wheat.

The aim of this study was the molecular and immunological characterization of new wheat allergens. In order to search for allergens that could be used as markers for certain clinical manifestations of wheat allergy, we screened a cDNA library prepared from wheat seed RNA with serum IgE from wheat allergic patients. The isolation and molecular characterization of five cDNAs coding for five wheat allergens (thioredoxin h, glutathione transferase, 1-Cys-peroxiredoxin, profilin and dehydrin), their expression and purification as soluble recombinant allergens in *Escherichia coli* is reported. Furthermore, the recombinant allergens were tested with sera from patients suffering from clinically defined forms of wheat allergy and their allergenic activity was studied in basophil degranulation experiments.

Methods

Biological materials

Wheat seeds (*T. aestivum*) were obtained from AGES (Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH, Vienna, Austria). Seeds from barley, maize, oat, rice, rye, spelt and sunflower were purchased at a local drugstore. Wheat pollen was obtained from Allergon (Välinge, Sweden), recombinant Phl p 1, Phl p 5, Phl p 7, Phl p 12 [17–19] from

BIOMAY (Vienna, Austria) and human serum albumin (HSA) from Behring (Marburg, Germany). *Escherichia coli* strain BL21 (DE3): F[−], ompTr_B[−]m_B[−] (DE) was purchased from Stratagene (La Jolla, CA, USA) and plasmid pET17b from Novagen (Madison, WI, USA).

Protein extracts

Proteins were extracted from 500 mg pollen and 15 g wheat seeds using 5 mL PBS, 2 mM EDTA, 1 mM PMSF at 4°C overnight. For the preparation of an aqueous extract, 15 g seeds were ground and extracted in 32 mL H₂O. The extracts were then centrifuged for 1 h at 13 000 g at 4°C.

A BCA Protein Assay Kit (Pierce, Rockford, IL, USA) was used to determine the concentration of the PBS wheat pollen extract (60 mg/mL), PBS wheat seed extract (20 mg/mL) and aqueous wheat seed extract (5 mg/mL), aliquots were stored at −20°C.

SDS-protein extracts for Western blotting [20] were prepared from barley, maize, oat, rice, rye, spelt, sunflower and wheat by homogenization of 3 g of tissue in 32 mL SDS-sample buffer. The powder was extracted by boiling for 10 min and centrifuged at 10 000 g for 10 min at 4°C. Extracts were stored at −20°C until use.

Sera

A wheat cDNA library was immunoscreened with serum IgE antibodies from four Spanish patients (A, B, C and D) suffering from wheat-induced respiratory allergy. Selection criteria were high levels of wheat-specific IgE antibodies (> 18 kUA/L), asthma during occupational exposure and positive skin tests [12].

Additional sera were obtained from 28 Italian patients (5 females, 23 males; mean age: 40 years; range: 20–61) and from eight Austrian patients (3 females, 5 males; mean age: 29; range: 12–46) who suffered from wheat-induced respiratory allergies. Each of them suffered from a clear history of occupational, wheat-induced symptoms such as asthma (10/36), rhinoconjunctivitis (21/36) or both (11/36). Patients were analysed regarding total serum IgE levels, wheat flour-specific IgE and rTri a 19 (i.e. omega-5-gliadin)-specific IgE by CAP-FEIA testing (Phadia, Uppsala, Sweden) (Table 1). The demographic, clinical and serological characterization of the patients is summarized in Table 1. Sera from 32 patients with wheat-induced food allergy have been described earlier [21]. These sera were used to test the frequency of IgE recognition of the recombinant wheat allergens in a population of patients with wheat-induced food allergy. Serum from a non-allergic person was used as negative control in all experiments. Sera were collected on the basis of a

Table 1. Demographic, clinical and serological characterisation of patients with respiratory allergy to wheat

Patient	Age	Sex	Wheat-induced symptoms	Wheat skin prick test (sqmm)	Wheat flour IgE (kUA/L)	Omega-5-gliadin (kUA/L)	Other allergies
1	48	m	RC	0.79	1.06	neg	c, hdm, m, mo, w
2	51	m	A, RC	7.07	4.69	neg	b, g, hdm, m
3	23	f	RC	neg	neg	neg	hdm
4	41	m	RC	12.56	neg	neg	hdm
5	33	m	A, RC	3.14	2.13	neg	b, c, g, hdm, o, w
6	61	m	RC	3.14	0.38	neg	g, hdm
7	52	m	A, RC	3.14	7.35	neg	b, hdm
8	34	f	RC	3.14	5.01	neg	b, c, g, hdm, m, o, w
9	33	m	A, RC	3.14	5.55	neg	g, hdm, m
10	58	m	RC	3.14	9.37	0.95	c, g, hdm, o
11	20	m	RC	12.56	1.92	neg	g, hdm, m, mo
12	22	m	A	3.14	2.28	neg	b, c, g, mo, w, y
13	30	f	A	7.07	3.99	neg	b, g, hdm, o
14	48	m	A, RC	3.14	9.89	1.13	hdm, o
15	53	m	A	3.14	4.08	neg	b, hdm, m, o
16	50	m	RC	3.14	2.44	neg	b, g, hdm, m, o
17	56	m	RC	7.07	1.76	neg	b, g, hdm, m, mo, o
18	21	m	RC	neg	4.93	neg	b, g, hdm, m, o, w
19	24	f	A, RC	3.14	3.85	neg	g, hdm
20	60	f	A, RC	28.26	6.43	neg	hdm
21	39	m	RC	3.14	5.18	neg	hdm
22	32	m	A	3.14	3.22	neg	b, g, hdm
23	27	m	RC	7.07	9.73	neg	neg
24	32	m	A	28.26	3.39	neg	b, c, g, hdm, m, o
25	61	m	A, RC	7.07	1.58	neg	b, hdm
26	32	m	RC	7.07	3.33	neg	neg
27	26	m	A	7.07	8.88	neg	b, g, hdm
28	52	m	RC	12.56	4.07	neg	b, hdm, g, mo, y
29	36	m	RC	neg	4.76	neg	g
30	35	m	A, RC	n.d.	24.3	n.d.	n.d.
31	29	f	A, RC	neg	9.75	n.d.	hdm, g
32	46	f	RC	neg	7.13	n.d.	neg
33	28	f	RC	n.d.	19.6	n.d.	n.d.
34	27	m	A, RC	n.d.	7.86	n.d.	n.d.
35	22	m	A	neg	13.1	n.d.	b, hdm, g
36	12	m	RC	pos	4.05	n.d.	b, hdm, g

m, male; f, female; kUA/L, kilounit antigen per litre; A, asthma; RC, rhinoconjunctivitis; b, birch pollen; c, cat; g, grass pollen; hdm, house dust mite; m, mugwort; mo, molds; o, olive pollen; w, weed pollen; y, yeast; n.d., not done.

study protocol approved by the IDI-IRCCS institutional review board, and the serological analysis was performed on anonymized sera with permission of the Ethics committee of the Vienna General Hospital, Vienna, Austria.

Rabbit antibodies specific for the newly isolated allergens were raised by immunization of rabbits with purified recombinant allergens (200 µg per injection) using once Freund's complete adjuvant and twice with incomplete Freund's adjuvant (Charles River, Kisslegg, Germany). Pre-immune sera were obtained from the rabbits before immunization.

Cloning, characterization, expression and purification of immunoglobulin E-reactive clones

A wheat seed cDNA library was constructed and screened with a serum pool from patients suffering from wheat allergy as described [12]. IgE-reactive phage clones, designated clone 37 (accession no. EU584496), clone 38 (accession no. EU584497), clone 112 (accession no. EU584498), clone 123 (accession no. EU584499) and clone 126 (accession no. EU584500) were purified to homogeneity by several rounds of IgE-re-screening. The cDNAs coding for the wheat allergens

were obtained by PCR amplification from the purified phage using lambda gt11 forward and reverse primers and directly sequenced (MWG, Ebersberg, Germany) [12]. (Table 2) The DNA and deduced amino acid sequences of the clones were compared with the sequences deposited in the GenBank database at the National Center for Biotechnology Information (NCBI). A sequence analysis was performed using the Clustal W multiple alignment tool.

The coding regions of the allergen-encoding cDNAs were PCR amplified using the primers (MWG) listed in Table 2. The primers contained *EcoRI* (underlined) and *NdeI* (italics) sites and a sequence coding for a C-terminal hexahistidine tag (bold). The amplified cDNAs were cut with *EcoRI* and *NdeI* and subcloned into plasmid pET17b [12].

Recombinant allergens, clone #37/thioredoxin h, clone #38/glutathione transferase, clone #112/1-Cys-peroxiredoxin, clone #123/profilin and clone #126/dehydrin were expressed in *E. coli* BL21 (DE3) and purified by nickel affinity chromatography from the soluble fraction of *E. coli* extracts (Quiagen; Hilden, Germany) [12]. Proteins were dissolved and stored in 10 mM NaH₂PO₄ buffer pH 7.5 at -20°C. The concentrations of the purified allergens were determined by BCA assay (Pierce). The purity of the recombinant proteins (3 µg/slot/20 µL) was checked by 14% SDS-PAGE and Coomassie Blue staining [20] and their identity was confirmed by Western blotting using a

monoclonal anti-His tag antibody (Novagen). Laser desorption mass spectra were acquired for the allergens in a linear mode with a TOF Compact MALDI II instrument (Kratos, Manchester, UK; piCHEM; Research and Development, Graz, Austria) [12].

Circular dichroism measurements were performed with recombinant wheat allergens with a concentration of 0.1 mg/mL on a Jasco J-810 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) using a 0.2 cm path length quartz cuvette, measured from 260 to 180 nm, with 0.5 nm resolution at a scanning speed of 50 nm/min and resulted from averaging of three scans. The final spectra were baseline corrected by subtracting the corresponding buffer spectrum. Results were expressed as the mean residue ellipticity [θ] at a given wavelength.

Purification of natural wheat allergens

Natural wheat allergens were purified by affinity chromatography from a PBS wheat seed extract using Protein G-purified rabbit IgG antibodies (ImmunoPure IgG purification Kit; Pierce). 3 mg of protein G-purified antibodies were coupled to a 1 mL HiTrapTM NHS-activated HP column (GE Healthcare Bio-Science AB, Uppsala, Sweden). Then 100 mg (5 mL of 20 mg/mL) of PBS wheat seed extract pH 7 was loaded, the column was then washed with PBS and bound allergens were eluted with 100 mM Glycine/HCl pH 2.8. Eluted fractions, containing purified natural allergens were pooled, concentrated (using Amicon Ultra, 10K; Millipore, Billerica, MA, USA) and dialysed against 10 mM NaH₂PO₄ pH 7. Purified natural allergens were separated by 14% SDS-PAGE (3 µg/slot/20 µL) and Silver-stained. The protein concentration was measured using a Micro BCA Assay Kit (Pierce).

Table 2. Primers used for PCR amplification and sub-cloning into the expression vector pET17b. *EcoRI* (underlined) and *NdeI* (italics) sites are indicated, the sequence coding for a C-terminal hexahistidine tag is printed bold

Primer	Sequence
37 fwd	5'-CAT ATG GCC GCC GAG GAG GGA GCC GTG ATA-3'
37 rev	5'-GAATTC TTA GTG ATG GTG ATG GTG ATG GGC AGA TGC AGA ACC-3'
38 fwd	5'-CAT ATG GCG GGC GAG AAG GGC CTG GTG CTG-3'
38 rev	5'-GAATTC TTA GTG ATG GTG ATG GTG ATG CTC GAT GCC GTA CTT-3'
112 fwd	5'-CAT ATG CCG GGC CTC ACC ATC GGC GAC ACC GTC-3'
112 rev	5'-GAATTC TTA GTG ATG GTG ATG GTG ATG GAC CTT GGT GAA GCG-3'
123 fwd	5'-CAT ATG TCG TGG CAG ACG TAC GTC GAC GAC-3'
123 rev	5'-GAATTC TTA GTG ATG GTG ATG GTG ATG GAA ACC CTG CTC GAC-3'
126 fwd	5'-CAT ATG GCG GAC TAC GGT GGA GAG TAC GGG-3'
126 rev	5'-GAATTC TTA GTG ATG GTG ATG GTG GTG TCC AGG GAG CTT-3'

Immunoglobulin E reactivity by dot-blotting, detection of cross-reactive allergens

Recombinant wheat proteins, recombinant grass pollen allergens (0.5 µg/dot), wheat pollen extract (3 µg/dot), wheat seed extract (2 µg/dot) and HSA (0.5 µg/dot) were dotted onto nitrocellulose strips (Schleicher & Schuell, Dassel, Germany). Nitrocellulose membranes were incubated with 1 : 10 diluted sera. Bound IgE antibodies were detected with ¹²⁵I-labelled anti-human IgE antibodies (Demeditec Diagnostics, Kiel, Germany) [20]. To define a cut-off for a positive/negative dot blot result, a non-allergenic protein HSA and serum from a non-allergic patient were used for control purposes. A reaction was considered positive when it was clearly visible at an exposure time when no background reactivity occurred with the negative controls (i.e. no reaction of allergic patients' sera with HSA and no reaction of the serum from the non-allergic individual to any of the tested antigens).

Nitrocellulose-blotted protein extracts from barley, maize, oat, rice, rye, spelt, sunflower and wheat were exposed to rabbit antibodies raised against the purified recombinant allergens or pre-immune sera (dilution 1 : 10 000) [20]. Bound rabbit antibodies were detected with 1 : 10 000 diluted ^{125}I -labelled anti-rabbit antibodies from donkey (GE Healthcare) and visualized to Kodak XOMAT films with intensifying screens (Kodak, Heidelberg, Germany) at -70°C [20].

Allergens in extracts from barley, maize, oat, rice, rye, spelt, sunflower showing IgE-cross-reactivity with wheat 1-Cys-peroxiredoxin were detected by IgE-immunoblot inhibition experiments [20].

Immunoglobulin E ELISA

ELISA plates (Nunc, Roskilde, Denmark) were coated with 0.5 μg of recombinant or natural wheat allergen or 5 μg of the aqueous wheat seed/pollen extract. After blocking with 1% (w/v) BSA in PBS, 0.05% (v/v) Tween 20 (PBST), plates were incubated with patients' sera (patients 29–36, B, C; Table 1) diluted 1 : 5 in PBST, 0.5% (w/v) BSA for measurement of IgE antibodies. Bound IgE antibodies were detected with an alkaline phosphatase-coupled mouse monoclonal anti-human IgE antibody (1 : 1000; Becton & Dickinson, Franklin Lakes, NJ, USA) and the colour reaction was developed with the 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma Aldrich, St. Louis, MO, USA). Measurements for comparing recombinant and natural allergens were performed in triplicates at OD 405–490 nm. Results are shown as means $\pm$ SD.

Basophil degranulation experiments

Rat basophil leukaemia (RBL) cells transfected with human Fc ϵ RI were incubated with sera from those patients with IgE reactivity to the recombinant allergens and wheat extract [22]. For control purposes, serum from a non-allergic person was used. Degranulation of the cells was induced by adding different concentrations of wheat seed extract (1000, 100, 10 ng/mL), of recombinant allergens (100, 10, 1 ng/mL) or buffer as a control. Released β -hexosaminidase from RBL cells exposed to allergen/IgE preparations was measured as described [23].

Results

Isolation of cDNAs coding for wheat allergens belonging to distinct protein classes

We used a serum pool from patients suffering from IgE-mediated wheat allergy [12] for screening of a cDNA library prepared from wheat seeds. Figure S1 shows the

individual reactivity profiles of three of the sera which had been used for IgE immunoscreening of the cDNA library (patients A, B and C). The comparison of the deduced amino acid sequences of IgE-reactive phage clones with published sequences showed that clone 37 is a wheat thioredoxin h. Thioredoxin h, designated Tri a 25, has been described as wheat allergen earlier [15, 24]. It has a calculated molecular weight of 13.5 kDa, shares the highest degree of sequence identity with thioredoxins from rice (73%) and maize (65%) and also exhibits sequence identities of more than 50% with thioredoxins from several other plant sources (e.g. Balsam poplar 57%; *Arabidopsis thaliana* 57%) (Fig. S2). However, the wheat thioredoxin h isolated by us seems to be an isoform which is substantially different from Tri a 25 because it has only 55% sequence identity with the earlier described Tri a 25. Wheat thioredoxin contains the highly conserved active site WCGPC (bold letters) and several amino acids which are supposed to maintain the tertiary structure of these proteins (asterisks) [25, 26].

The cDNA of clone 38 codes for wheat glutathione transferase, a protein of 25.8 kDa which shares a high degree of sequence identity with glutathione transferases from barley (95%), maize (67%), oil palm (65%), rice (63%) and soy (59%) (Fig. S3). Clone 112 codes for wheat 1-Cys-peroxiredoxin a protein with a deduced molecular weight of 24.8 kDa (Fig. S4). It shows a more than 80% sequence identity with homologues from barley, rye, rice and maize and a more than 50% sequence identity with homologues in dicotyledonic plants (e.g. sunflower, rape) (Fig. S4).

Thioredoxin, glutathione transferase and 1-Cys-peroxiredoxin belong to a group of proteins with reducing activity which resemble typical thioredoxin-like folds in their three-dimensional structure (Figs S2–S4, Table 3) [26, 27].

Clone 123 codes for wheat profilin, a cytoskeletal protein which has been described as a highly conserved cross-reactive allergen [20, 28]. It has a deduced molecular weight of 14.9 kDa and shares more than 83% amino acid sequence identity with other plant profilins (Table 3).

Clone 126 codes for wheat dehydrin, a protein with a deduced molecular weight of 22.4 kDa which shares a sequence identity of more than 50% with dehydrins from barley, rice and oil palm (Fig. S5, Table 3). Dehydrins represent proteins containing two typical dehydrin sequence motifs (Fig. S5) whose expression is up-regulated upon environmental stress such as lack of water [29].

Expression and purification of recombinant wheat allergens, affinity purification of natural allergens

We expressed the wheat allergens as recombinant proteins with a C-terminal hexahistidine tag in *E. coli*.

Table 3. Groups of wheat allergens and their amino acid sequence identities.

Group	Wheat clone	Allergen name	Homologous proteins/allergen source/%sequence identity
Thioredoxin-like fold	#37	Thioredoxin h	Rice (73%), maize (65%), poplar (57%), thale cress (57%), eucalyptus (56%), sage (55%), ricinus (55%), citrus (55%), chilli (54%), peach tree (53%), soy (53%), clover (52%), sweet potato (52%), greater plantain (52%)
	#38	Glutathione transferase	Barley (95%), maize (67%), oil palm (65%), rice (63%), soy (59%), papaya (58%), mallow (58%), vine (57%), pea (56%), ricinus (56%), tobacco (55%), camomille (55%), chilli (55%), thale cress (52%)
	#112	1-Cys-peroxiredoxin	Barley (99%), rye (96%), rice (86%), maize (83%), sunflower (74%), poplar (72%), ricinus (72%), thale cress (72%), barrel clover (71%), buckwheat (71%), oil palm (70%), rape (70%), bog (70%), zebra finch (58%)
Cytoskeletal protein	#123	Profilin	Maize (83%), cinnamon (80%), oil palm (80%), soy (80%), olive tree (79%), parsley (78%), coconut palm (78%), grass (78%)
Stress-induced protein	#126	Dehydrin	Barley (76%), rice (58%), oil palm (54%)

The recombinant allergens were purified using nickel affinity chromatography from the supernatants of lysed *E. coli* as soluble proteins (Fig. 1). Natural allergens were isolated from PBS wheat seed extract by affinity chromatography using rabbit antibodies raised against the purified recombinant allergens. Figure 1a shows a silver-stained 14% SDS-PAGE [30] containing the purified natural wheat allergens which showed comparable molecular weights as the corresponding recombinant allergens (Fig. 1b). Only in case of dehydrin additional bands which may represent oligomers or degradation products were observed in the natural purified allergens. Figure 1b demonstrates the purity and migration of the recombinant allergens, i.e. thioredoxin h

(13 kDa), glutathione transferase (25 kDa), 1-Cys-peroxiredoxin (25 kDa), profilin (14 kDa) and dehydrin (35 kDa) and the protein composition of wheat extracts (i.e. aqueous extract, PBS extract, SDS extract) using a Coomassie brilliant blue-stained SDS-PAGE. The yields of purified recombinant allergens/L culture were as follows: thioredoxin: 1.7 mg/L; glutathione transferase: 1 mg/L; 1-Cys-peroxiredoxin: 44 mg/L; profilin: 16.1 mg/L; dehydrin: 34.4 mg/L.

The results of the MALDI-TOF analysis of purified recombinant proteins corresponded with the deduced molecular weights indicating that the recombinant proteins do not contain a methionine, except the recombinant profilin (data not shown). Circular dichroism analysis of recombinant allergens showed that all allergens except dehydrin represented folded proteins (Fig. 2).

Occurrence of cross-reactive proteins in seeds from various plants

Using rabbit antibodies specific for recombinant 1-Cys-peroxiredoxin from wheat, we detected cross-reactive proteins in seed extracts from barley, rye, rice, maize, soy, oat and spelt but not in sunflower (data not shown). When serum from a patient with IgE reactivity to wheat 1-Cys-peroxiredoxin was pre-incubated with the recombinant wheat allergen, IgE reactivity to cross-reactive allergens at 20–25 kDa in seed extracts from barley, rye, rice, maize, soy, oat and spelt was abolished or reduced compared to pre-incubation with the unrelated birch pollen allergen, Bet v 1 (Fig. 3).

Using rabbit antibodies raised against purified recombinant wheat thioredoxin h, glutathione transferase, dehydrin and profilin, we searched for cross-reactive allergens in the seed extracts. Thioredoxin h was detected in rice, maize, sunflower and spelt, glutathione transferase in oat and spelt, wheat profilin-specific antibodies identified profilin in maize, rice, oat and rye and dehydrin was identified in rice, maize, soy, oat, rye and spelt (data not shown).

Identification of recombinant wheat allergens which are specifically recognized by immunoglobulin E from patients with respiratory wheat allergy

Figure 4 shows the reactivity of dot-blotted recombinant wheat and grass pollen allergens with IgE from 28 patients suffering from wheat-induced respiratory allergy (Table 1). Each of these patients exhibited IgE reactivity to dot-blotted wheat seed extract. Glutathione transferase (10.7%), profilin (10.7%), 1-Cys-peroxiredoxin (35.7%) and dehydrin (7.1%) were recognized to a varying degree. 1-Cys-peroxiredoxin was recognized by serum IgE from 35.7% of the tested patients and

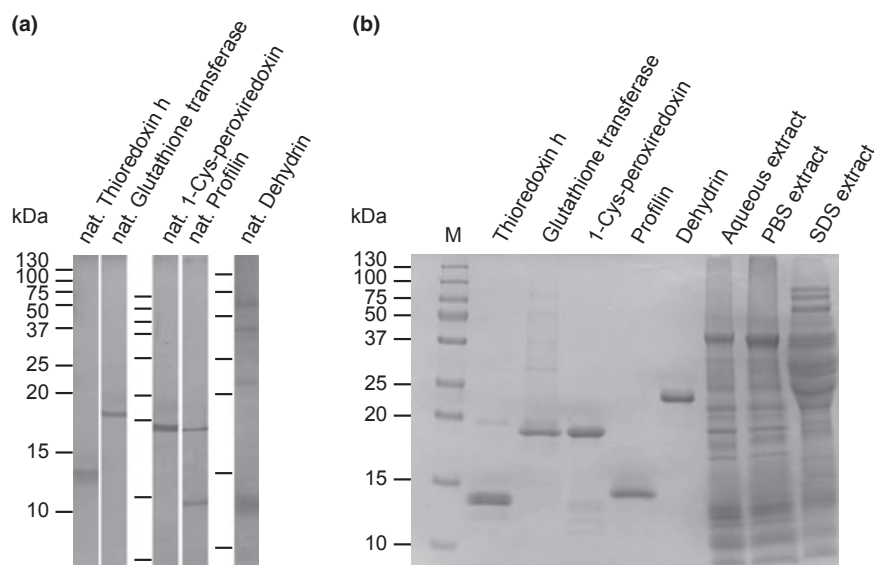


Fig. 1. Comparison of recombinant and natural wheat allergens. (a) Silver-stained SDS-PAGE containing purified natural wheat allergens and (b) Coomassie brilliant blue-stained SDS-PAGE containing purified recombinant wheat allergens, aqueous extract, PBS extract and SDS wheat extract. Molecular weights (kDa) are indicated at the left margin.

thus was the most frequently recognized allergen in our patient population (Fig. 4, lower panel).

Weak dot blot signals could be confirmed by additional IgE ELISA experiments for sera where sufficient volumes were available (the OD values corresponding to bound IgE for these sera and antigens were as follows: Phl p 1, sera #5: 2.113; #22: 0.949; buffer control: 0.044; wheat pollen extract, sera #9: 3.954; #10: 3.025; #12: 3.959; #14: 3.962; buffer control: 0.132; wheat seed extract, sera #8: 1.261; #13: 1.208; #18: 0.727; #21: 1.627; #26: 0.851; buffer control: 0.333; dehydrin, sera #7: 0.986; #24: 2.953; buffer control: 0.075). None of the tested sera showed IgE reactivity to recombinant thioredoxin that has been identified as allergen recognized by patients with wheat-induced food allergy. Recombinant wheat seed profilin reacted with sera from patients who were also allergic to grass pollen and who exhibited also IgE reactivity to recombinant timothy grass pollen profilin, rPhl p 12. With the combination of recombinant glutathione transferase, profilin, 1-Cys-peroxiredoxin and dehydrin 50% of patients could be detected. Weak IgE reactivity (< 1.5 kUA/L) to rTri a 19 (i.e. omega-5-gliadin) was detected in only two of the 28 sera (Table 1).

Recombinant timothy grass pollen allergens which represent markers for a genuine grass pollen sensitization (i.e. rPhl p 1, rPhl p 5) and highly cross-reactive timothy grass pollen allergens (i.e. rPhl p 7, rPhl p 12) were included in the analysis [21]. Patients suffering also from grass pollen allergy showed IgE reactivity to the recombinant timothy grass pollen allergens and wheat pollen extract (Fig. 4, upper panel).

None of the patients exhibited IgE reactivity to dot-blotted HSA and no reactivity to any of the dotted proteins was observed with serum from the non-allergic person (Fig. 4). None of 32 sera from patients with wheat food allergy showed IgE reactivity to thioredoxin h, glutathione transferase, 1-Cys-peroxiredoxin or dehydrin (data not shown).

A comparison of the IgE reactivity of natural and recombinant allergens could be made for those allergens (i.e. glutathione transferase, 1-Cys-peroxiredoxin, profilin) for where positive serum samples were available (Tables 1 and 4). Each of the sera which contained IgE antibodies to the natural allergens also reacted with the corresponding recombinant allergens, albeit with sometimes different intensities. IgE reactivity of recombinant #38 glutathione transferase was lower than that of the natural allergen, whereas recombinant #112 1-Cys-peroxiredoxin and recombinant #123 profilin showed higher IgE reactivity compared with the natural allergens in the majority of positive sera.

Recombinant wheat allergens show varying immunoglobulin E-reactivity and allergenic activity

To investigate the allergenic activity of the individual recombinant allergens, RBL cells expressing the human FcεRI were loaded with serum IgE from the patients with wheat-induced respiratory allergy and exposed to different concentrations of the recombinant allergens, wheat seed extract or buffer as a control (Fig. 5).

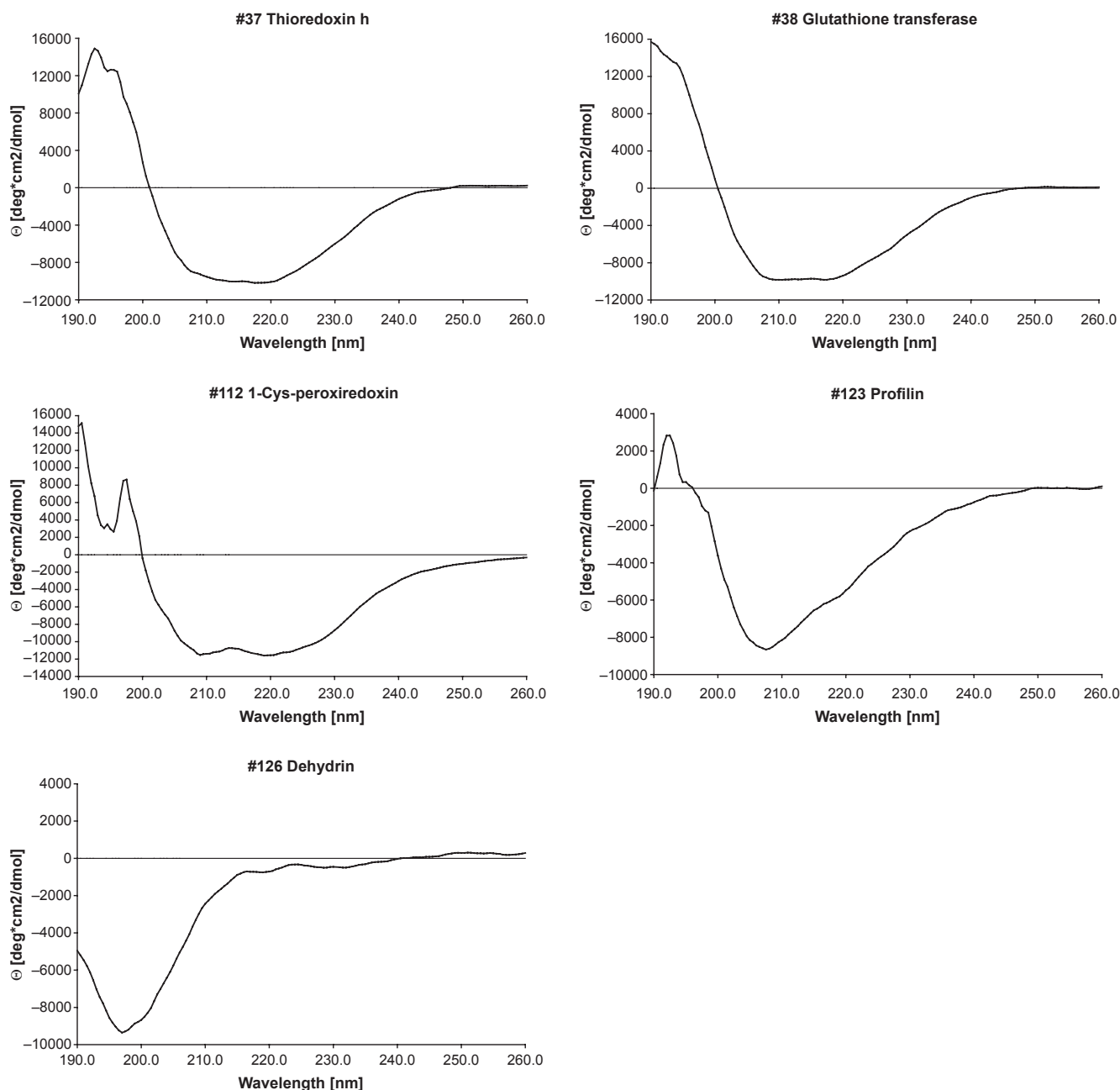


Fig. 2. CD spectra of purified recombinant wheat allergens, #37 thioredoxin h, #38 glutathione transferase, #112 1-Cys-peroxiredoxin, #123 profilin, and #126 dehydrin. The mean residue ellipticities (Θ) (y-axes) are shown at given wavelengths (x-axes).

Glutathione transferase induced specific and dose-dependent degranulation with sera from the two patients (Fig. 5: #2, 10) who had shown strong IgE reactivity in the dot blot (Fig. 4). Also, 1-Cys-peroxiredoxin induced specific and dose-dependent degranulation with sera that had shown the strongest IgE reactivity to the dot-blotted allergen (Fig. 4: #3, 6, 19, 22) but not with the other sera with weak IgE reactivity (Fig. 4: #2, 4, 12, 13, 15, 27) (data not shown). Recombinant wheat

dehydrin and profilin did not induce relevant basophil degranulation (data not shown). Each of the tested sera except serum #6 induced basophil degranulation with wheat extract. However, serum #6 had induced degranulation with recombinant 1-Cys-peroxiredoxin (Fig. 5).

It thus appeared that those allergens which exhibited strong IgE reactivity were also more potent in inducing basophil activation than those with lower IgE binding capacity.

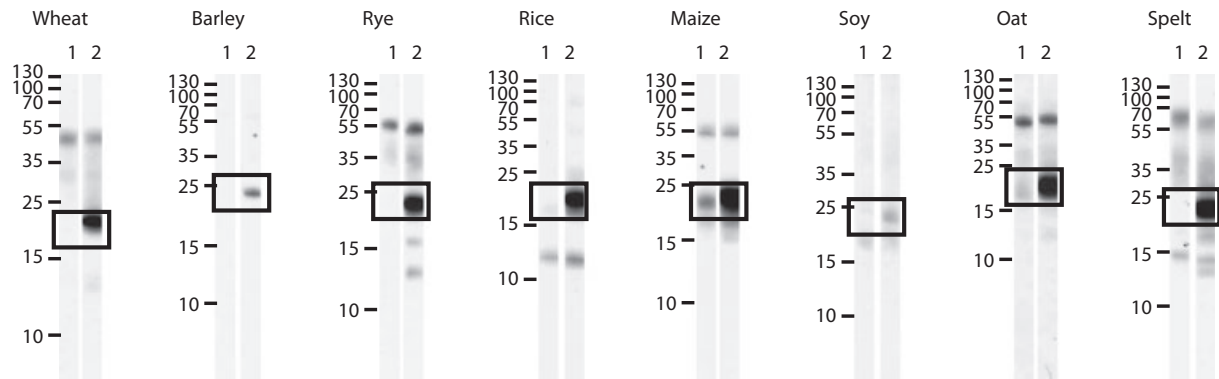


Fig. 3. Detection of plant seed allergens with IgE cross-reactivity to wheat 1-Cys-peroxiredoxin. Serum from a patient with IgE reactivity to wheat 1-Cys-peroxiredoxin was pre-incubated with recombinant wheat 1-Cys-peroxiredoxin (lanes 1) or rBet v 1 (lanes 2) and exposed to nitrocellulose-blotted seed extracts from wheat, barley, rye, rice, maize, soy, oat, spelt. Bound IgE antibodies were detected and visualized by autoradiography. Molecular weights (kDa) are displayed on the left margins.

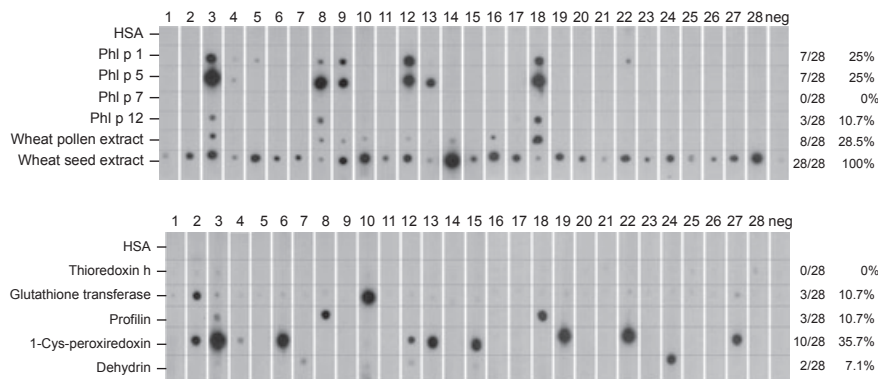


Fig. 4. IgE reactivity of patients suffering from respiratory wheat allergy. Dot-blotted purified recombinant wheat proteins (thioredoxin h, glutathione transferase, profilin, 1-Cys-peroxiredoxin, dehydrin), human serum albumin, recombinant grass pollen allergens (rPhl p 1, rPhl p 5, rPhl p 7, rPhl p 12) and wheat pollen and seed extract were incubated with sera from 28 patients suffering from respiratory wheat allergy (1–28) and with serum from one non-allergic individual (neg). Bound IgE Abs were detected with ^{125} I-labelled anti-human IgE Abs and visualized by autoradiography. The numbers and percentages of reactive sera are displayed on the right margin.

Table 4. Comparison of IgE reactivity of recombinant and natural wheat allergens. ELISA plate-bound natural and recombinant wheat allergens, #38 glutathione transferase, #112 1-Cys-peroxiredoxin and #123 profilin were incubated with patient's sera (29–36, B, C) or serum from a non-allergic individual (N). The mean OD values $\pm$ SDs corresponding to the levels of specific IgE are displayed. Due to shortness of sera, IgE reactivity of natural and recombinant allergens could be compared only for certain patients.

Patient	OD values					
	Glutathione transferase		1-Cys-peroxiredoxin		Profilin	
	nat#38	rec#38	nat#112	rec#112	nat#123	rec#123
29	0.81 $\pm$ 0.056	0.259 $\pm$ 0.0311	n.d.	n.d.	n.d.	n.d.
30	n.d.	n.d.	0.23 $\pm$ 0.007	1.478 $\pm$ 0.045	n.d.	n.d.
31	n.d.	n.d.	0.103 $\pm$ 0.004	1.528 $\pm$ 0.064	n.d.	n.d.
32	n.d.	n.d.	0.247 $\pm$ 0.006	1.593 $\pm$ 0.076	n.d.	n.d.
33	n.d.	n.d.	n.d.	n.d.	0.407 $\pm$ 0.005	0.497 $\pm$ 0.02
34	n.d.	n.d.	n.d.	n.d.	1.56 $\pm$ 0.063	1.03 $\pm$ 0.129
35	n.d.	n.d.	n.d.	n.d.	0.289 $\pm$ 0.023	1.419 $\pm$ 0.098
36	n.d.	n.d.	n.d.	n.d.	0.139 $\pm$ 0.008	1.095 $\pm$ 0.071
B	0.34 $\pm$ 0.016	0.077 $\pm$ 0.005	0.169 $\pm$ 0.032	0.281 $\pm$ 0.007	n.d.	n.d.
C	n.d.	n.d.	1.11 $\pm$ 0.018	0.174 $\pm$ 0.016	1.62 $\pm$ 0.021	2.267 $\pm$ 0.101
N	0.049 $\pm$ 0.004	0.044 $\pm$ 0.002	0.07 $\pm$ 0.017	0.042 $\pm$ 0.002	0.056 $\pm$ 0.021	0.05 $\pm$ 0.003

n.d., not done.

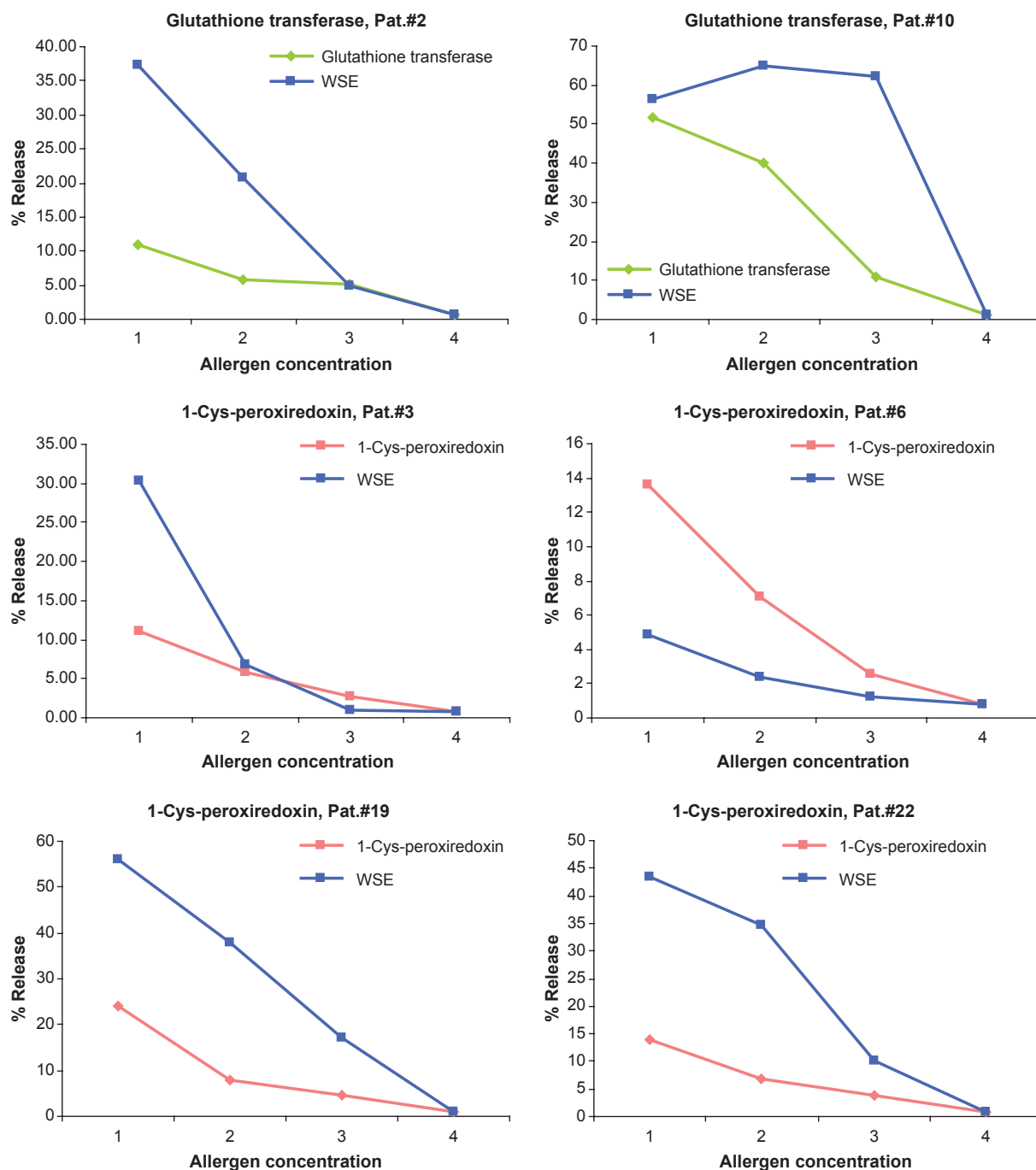


Fig. 5. Allergenic activity of recombinant allergens. RBL cells transfected with the human Fc ϵ RI loaded with sera from patients with respiratory wheat allergy containing allergen-specific IgE were incubated with wheat seed extract (WSE) (concentrations 1 : 1000, 2 : 100, 3 : 10 ng/mL), recombinant wheat allergens (concentrations 1 : 100, 2 : 10, 3 : 1 ng/mL) or buffer alone (concentration 4 : 0 ng/mL). β -hexosaminidase releases are displayed as percentages of total β -hexosaminidase on the y-axes.

Discussion

Here we report the molecular characterization of five wheat allergens as well as their production and evaluation as recombinant allergens. According to sequence analysis, the allergens belong to three groups of proteins. The first three allergens (i.e. thioredoxin h, glutathione transferase and 1-Cys-peroxiredoxin) form a

group of proteins assembling a thioredoxin-like fold. They have general reducing activity and thus act as antioxidants. A thioredoxin h isoform with only moderate (55%) sequence identity has in fact been described earlier as a major wheat allergen [15, 24]. Wheat profilin belongs to a family of cytoskeletal

proteins which sequester actin and participate in signal transduction. Profilins have been identified as ubiquitous and cross-reactive allergens in numerous plant species and plant tissues [20]. Finally, dehydrin represents a protein which is up-regulated in plants upon lack of water and thus functions as a stress protein which should protect against denaturation.

Each of the five allergens could be expressed with good yield (> 1 mg/L culture) in *E. coli* as soluble proteins which allowed us to perform a serological evaluation of the frequency of IgE recognition of the allergens in a population of patients suffering from wheat-induced respiratory allergy. We have also tested the frequency of IgE reactivity of the recombinant wheat allergens in a microarray format in 32 wheat food allergic patients, 22 more patients suffering from baker's asthma and in 17 grass pollen allergic patients [21]. The results from our study and the earlier microarray studies demonstrate that thioredoxin h, glutathione transferase, 1-Cys-peroxiredoxin and dehydrin are mainly recognized by sera from patients suffering from wheat-induced respiratory allergy but not from patients with wheat-induced food allergy.

This result is quite surprising considering that homologous proteins were detected in seeds from other plant species using IgG antibodies raised against the purified recombinant allergens and by IgE cross-reactivity in the case of 1-Cys-peroxiredoxin. Furthermore, sequence identities of more than 70% were found in homologous proteins from other plants, suggesting that they may act as cross-reactive allergens [31].

A possible explanation for the rather selective recognition by patients suffering from respiratory symptoms may be that these proteins are more easily digested than wheat allergens involved in plant food allergy. Among the recombinant allergens identified, 1-Cys-peroxiredoxin was recognized by IgE antibodies from more than 30% of the patients with wheat-induced respiratory allergy and thus appeared to be the most frequently recognized allergen, followed by glutathione transferase and dehydrin. Profilin represents a cross-reactive allergen which also reacts with IgE antibodies from grass pollen allergic patients. It was recognized by approximately 10% of the patients. Tri a 25, a thioredoxin which has 55% sequence homology with the thioredoxin isolated by us, has been reported as a major wheat allergen for baker's asthma. However, the recombinant thioredoxin h isolated in our study reacted only with one serum in the serum pool used for screening. The latter may be explained by the fact that it represents a distantly related and perhaps poorly allergenic thioredoxin h isoform. A comparison of the recombinant allergens with purified natural proteins showed that sera reacted with the recombinant as well as with the natural allergens but sometimes with different intensities which may be explained rather by the pres-

ence of different isoforms than by structural characteristics, because all but one (i.e. dehydrin) of the recombinant allergens represented folded proteins. The lower IgE reactivity of recombinant vs. natural glutathione transferase may be due to lack of glycosylation of the recombinant *E. coli*-expressed protein because one glycosylation site was predicted by sequence analysis in this protein. Nevertheless, specific allergenic activity could be demonstrated for recombinant glutathione transferase and 1-Cys-peroxiredoxin in basophil degranulation experiments.

Current serological tests based on wheat flour extracts do not discriminate between patients suffering from wheat food allergy or other wheat-induced forms of allergy and may yield false-positive results due to the presence of broadly cross-reactive allergens such as profilin and IgE-reactive carbohydrate moieties [32]. Serological tests containing recombinant wheat allergens may be used to identify patients suffering from certain manifestations of wheat-associated allergy.

In fact, according to serology and evaluation of allergenic activity in basophil release assays, we could identify glutathione transferase and in particular 1-Cys-peroxiredoxin as relevant wheat allergens for patients suffering from respiratory allergy to wheat.

Using the latter two allergens and a recently described serine protease inhibitor, it may be possible to establish serological tests for the identification of patients suffering from baker's asthma. In addition, other recently characterized allergens such as the glutenins and the wheat LTP [33] and non-wheat-derived allergens may be included in a test for the identification of baker's asthma patients. Such tests may be of clinical relevance for IgE-based serological screening to identify persons who have developed an occupational sensitization to wheat flour allergens such as bakers and persons working in food industry and restaurants.

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Conflict of interest: Josef Thalhamer has acted as a paid consultant to Biomay (Vienna). He has received funding for research carried out in this work. Rudolf Valenta has acted as a paid consultant for Phadia, Uppsala, Sweden, and has received funding for research carried out in this work.

References

- 1 Tatham AS, Shewry PR. Allergens to wheat and related cereals. *Clin Exp Allergy* 2008; 38:1712–26.
- 2 Asero R, Antonicelli L, Arena A et al. Causes of food-induced anaphylaxis in Italian adults: a multi-centre study. *Int Arch Allergy Immunol* 2009; 150: 271–7.
- 3 Hur GY, Koh DH, Kim HA et al. Prevalence of work-related symptoms and serum-specific antibodies to wheat flour in exposed workers in the bakery industry. *Respir Med* 2008; 102: 548–55.
- 4 Zuidmeer L, Goldhahn K, Rona RJ et al. The prevalence of plant food allergies: a systematic review. *J Allergy Clin Immunol* 2008; 121:1210–18 e4.
- 5 van Kampen V, Rabstein S, Sander I et al. Prediction of challenge test results by flour-specific IgE and skin prick test in symptomatic bakers. *Allergy* 2008; 63:897–902.
- 6 Jacquenet S, Morisset M, Battais F et al. Interest of ImmunoCAP system to recombinant omega-5 gliadin for the diagnosis of exercise-induced wheat allergy. *Int Arch Allergy Immunol* 2009; 149:74–80.
- 7 Matsuo H, Dahlstrom J, Tanaka A et al. Sensitivity and specificity of recombinant omega-5 gliadin-specific IgE measurement for the diagnosis of wheat-dependent exercise-induced anaphylaxis. *Allergy* 2008; 63:233–6.
- 8 Matsuo H, Morita E, Tatham AS et al. Identification of the IgE-binding epitope in omega-5 gliadin, a major allergen in wheat-dependent exercise-induced anaphylaxis. *J Biol Chem* 2004; 279:12135–40.
- 9 Palosuo K, Alenius H, Varjonen E et al. A novel wheat gliadin as a cause of exercise-induced anaphylaxis. *J Allergy Clin Immunol* 1999; 103:912–7.
- 10 Sandiford CP, Tatham AS, Fido R et al. Identification of the major water/salt insoluble wheat proteins involved in cereal hypersensitivity. *Clin Exp Allergy* 1997; 27:1120–9.
- 11 Bittner C, Grassau B, Frenzel K, Baur X. Identification of wheat gliadins as an allergen family related to baker's asthma. *J Allergy Clin Immunol* 2008; 121:744–9.
- 12 Constantin C, Quirce S, Grote M et al. Molecular and immunological characterization of a wheat serine proteinase inhibitor as a novel allergen in baker's asthma. *J Immunol* 2008; 180:7451–60.
- 13 Gomez L, Martin E, Hernandez D et al. Members of the alpha-amylase inhibitors family from wheat endosperm are major allergens associated with baker's asthma. *FEBS Lett* 1990; 261:85–88.
- 14 Palacin A, Varela J, Quirce S et al. Recombinant lipid transfer protein Tri a 14: a novel heat and proteolytic resistant tool for the diagnosis of baker's asthma. *Clin Exp Allergy* 2009; 39:1267–76.
- 15 Weichel M, Glaser AG, Ballmer-Weber BK, Schmid-Grendelmeier P, Cramer R. Wheat and maize thioredoxins: a novel cross-reactive cereal allergen family related to baker's asthma. *J Allergy Clin Immunol* 2006; 117:676–81.
- 16 Rihs HP, Rozynek P, May-Taube K, Welticke B, Baur X. Polymerase chain reaction based cDNA cloning of wheat profilin: a potential plant allergen. *Int Arch Allergy Immunol* 1994; 105: 190–4.
- 17 Andersson K, Lidholm J. Characteristics and immunobiology of grass pollen allergens. *Int Arch Allergy Immunol* 2003; 130:87–107.
- 18 Niederberger V, Laffer S, Froschl R et al. IgE antibodies to recombinant pollen allergens (Phl p 1, Phl p 2, Phl p 5, and Bet v 2) account for a high percentage of grass pollen-specific IgE. *J Allergy Clin Immunol* 1998; 101:258–64.
- 19 Kazemi-Shirazi L, Niederberger V, Linhart B, Lidholm J, Kraft D, Valenta R. Recombinant marker allergens: diagnostic gatekeepers for the treatment of allergy. *Int Arch Allergy Immunol* 2002; 127:259–68.
- 20 Valenta R, Duchene M, Ebner C et al. Profilins constitute a novel family of functional plant pan-allergens. *J Exp Med* 1992; 175:377–85.
- 21 Constantin C, Quirce S, Poorafshar M et al. Heberle-Bors E, Nystrand M, Valenta R. Micro-arrayed wheat seed and grass pollen allergens for component-resolved diagnosis. *Allergy* 2009; 64:1030–7.
- 22 Ladics GS, van Bilsen JH, Brouwer HM, Vogel L, Vieths S, Knippels LM. Assessment of three human FcepsilonRI-transfected RBL cell-lines for identifying IgE induced degranulation utilizing peanut-allergic patient sera and peanut protein extract. *Regul Toxicol Pharmacol* 2008; 51:288–94.
- 23 Schulmeister U, Hochwallner H, Swo-boda I et al. Cloning, expression, and mapping of allergenic determinants of alphaS1-casein, a major cow's milk allergen. *J Immunol* 2009; 182: 7019–29.
- 24 Weichel M, Vergoossen NJ, Bonomi S et al. Screening the allergenic repertoires of wheat and maize with sera from double-blind, placebo-controlled food challenge positive patients. *Allergy* 2006; 61:128–35.
- 25 Eklund H, Gleason FK, Holmgren A. Structural and functional relations among thioredoxins of different species. *Proteins* 1991; 11:13–28.
- 26 Copley SD, Novak WR, Babbitt PC. Divergence of function in the thioredoxin fold suprafamily: evidence for evolution of peroxiredoxins from a thioredoxin-like ancestor. *Biochemistry* 2004; 43:13981–95.
- 27 Martin JL. Thioredoxin – a fold for all reasons. *Structure* 1995; 3:245–50.
- 28 Valenta R, Duchene M, Pettenburger K et al. Identification of profilin as a novel pollen allergen; IgE autoreactivity in sensitized individuals. *Science* 1991; 253:557–60.
- 29 Close TJ, Kortt AA, Chandler PM. A cDNA-based comparison of dehydration-induced proteins (dehydrins) in barley and corn. *Plant Mol Biol* 1989; 13:95–108.
- 30 Sammons DW, Adams LD, Nishizawa EE. Ultrasensitive silver-based color stainings of polypeptides in polyacrylamide gels. *Electrophoresis* 1981; 2:135–41.
- 31 Aalberse RC. Assessment of allergen cross-reactivity. *Clin Mol Allergy* 2007; 5:2.
- 32 van Kampen V, Merget R, Rabstein S et al. Comparison of wheat and rye flour solutions for skin prick testing: a multi-centre study (Stad 1). *Clin Exp Allergy* 2009; 39:1896–902.
- 33 Palacin A, Quirce S, Armentia A et al. Wheat lipid transfer protein is a major allergen associated with baker's asthma. *J Allergy Clin Immunol* 2007; 120:1132–8.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. IgE reactivity of patient's sera used for IgE immunoscreening. Dot-blotted purified recombinant wheat proteins (1-Cys-peroxiredoxin, profilin, dehydrin, thioredoxin h, glutathione transferase), human serum albumin and wheat pollen extract were incubated with sera from four patients suffering from respiratory wheat allergy (patients A, B and C), with serum from one non-allergic individual (Neg) or buffer alone (Buffer). Bound IgE Abs were detected with ^{125} I-labelled anti-human IgE Abs and visualized by autoradiography.

Figure S2. Sequence alignment of the clone 37-derived allergen thioredoxin with homologous proteins in other plants. The amino acid sequence (single letter code) of wheat thioredoxin was aligned with thioredoxins from rice (gi|27461140), maize (gi|40287476), poplar (gi|74058514), thale cress (gi|80973754), eucalyptus (gi|116788626), sage (gi|118481453), ricinus (gi|119367477), citrus (gi|157781191), chilli (gi|195645418), peach tree (gi|208659912), soy (gi|115470941), clover (gi|186972814), sweet potato (gi|11135312) and greater plantain (gi|255587090). Identical amino acids are indicated by points, gaps (dashes) were introduced to improve the alignment. The conserved active site WCGPC is indicated in bold letters. Amino acids reported to be important for the maintenance of the tertiary structure and function are highlighted with asterisks (*).

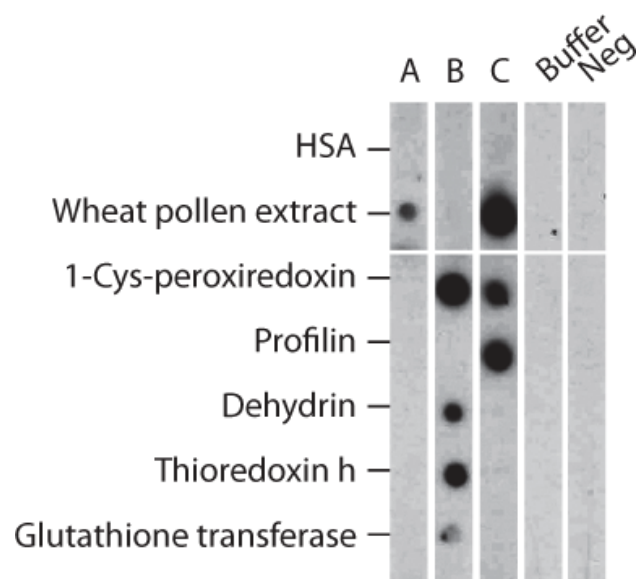
Figure S3. Sequence alignment of glutathione transferase, the clone 38-derived wheat allergen with homologous plant allergens. Alignment of wheat glutathione transferase sequence (top line) with sequences from barley (gi|6683765), maize (gi|195639794), oil palm (gi|

192911948), rice (gi|115471993), soy (gi|2920666), papaya (gi|2853219), mallow (gi|29419702), vine (gi|119633090), pea (gi|110749703), ricinus (gi|255584168), tobacco (gi|19817), camomille (gi|17385642), chilli (gi|58578272) and thale cress (gi|21592644). Identical amino acids are indicated by points, gaps (dashes) were introduced to improve the alignment.

Figure S4. Alignment of 1-Cys-peroxiredoxin, the clone 112-derived wheat allergen with homologous plant proteins. Alignment of the wheat 1-Cys-peroxiredoxin sequence (top line) with sequences from barley (gi|1710077), rye (gi|1710076), rice (gi|158517776), maize (gi|162460575), sunflower (gi|109631620), poplar (gi|224101487), ricinus (gi|255556526), thale cress (gi|28393058), barrel clover (gi|75323225), buckwheat (gi|6466096), oil palm (gi|192910660), rape (gi|7381260), bog (gi|1710079) and zebra finch (gi|197128384).

Figure S5. Alignment of dehydrin, the clone 126-derived wheat allergen with homologous plant proteins. Alignment of the wheat dehydrin sequence (top line) with sequences from barley (gi|6017948), rice (gi|115439431), oil palm (gi|7330252), thale cress (gi|30693389), grass (gi|2970213), grass (gi|121489509), coffee (gi|84314116), ricinus (gi|255561008), vine (gi|57903608), Mexican sunflower (gi|18076154), rape (gi|34539778), sawlow thorn (gi|33114013), sunflower (gi|14588999) and green tea (gi|215398978). Boxes marked in grey illustrate typical dehydrin sequence motifs.

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		***	*	*	*	*	*
Thioredoxin h	----	MAAEEGAVIACHTKQEF	DTHMANGKETGKLV	IDFTASWC	GPCRVIAPVFAEYAKK-FPGAIFLKVDVDELKDVAEAYNVEAMPTFLFIKDGA	KVDTVVGGRKDDIHTKIVALMGSASA	
Rice	-----	V....N.D...AQ.TKA..A.....F.....E...K.....EA.K...A....LQNT..KHV.ATA.SASA					73%
Maize	-----	S...V.....AD..A...KA..A.....F...L.V.H...-TQ.V.....E..A..D.....H.V.N.VT.E...A..ENLLAQ.EKHCAA.VPAA					65%
Poplar	-----	D.Q...G...VEAW.EQLQR.N.SK...V...A.....FL..L.R.-L.DV.....T..QDWA.....H..L.E.KI...K...A...ELQQA.AKHTAP.A.TASA					57%
Thale cress	-----	Q...S...NDVWTVQLDKA..SN..IV.....P...M...I.NDL...FMSS...F.....QS..KEFG.....V...A.EV...KL..AN.E.LQA...KHT.VTT.					57%
Eucalyptus	-----	M...Q..S...SAESWSEI.KSN.SD...VV.....F...FL..L.R-..NVL.....T..QEWA.....H.V.G.KI...R...AQ...QLQMTLAKH.AT...					56%
Sage	--MAS-V...	Q...S...SVD.WKE.FQK.VDSN...VV.....F...IL..I...-T.HV.....T..QE..I...S...E.KEI.R...A..E.LLA.VTQHG.A.LTASA					55%
Ricinus	-----	Q...G...VEAWNEQLQK.ND.KG.IVV.....F...FL..L...-L.NVT.....T..HEWA..S...H..L.E.KIM.K...AK...ELQQT.AKH.ATA.T					55%
Citrus	---MA....	Q..G...VEAWNEQLQKSN..KQ..VV.....F...FL..L...-L.NVL.....S..TDWA.....H..L.E.KI...K...SK.EELQQT.AKHLAT..T					55%
Chili	-MAATSS...	Q.FG...NVE.W.Q.FKK.V..K...VV.....F...IL..DI...-M.HV.....T...EW..D....V.F...EE...R...AQ.EELQAA.LKHVGAPATVTA					54%
Peach tree	-----	M...NQ..G...T.A.EEQLHK.N.NK...VV.....L...IL..L...-T.EVT.....RT.S.EWG.....L.E.KI...K...AK...ELQI.VAKHVAA.A.ASAT					53%
Soy	--MAGSS...	Q..S...VE.WNDQLQK.N.SK..IVV.....F...FL..L...-T.SV.....S.SQDWAI....V.V.E.TLL.K...AK...ELQQ...QKHVA.SN.					53%
Clover	-----	Q...GV...VEQNKEEIQK.NDSK..IVV.....F...IL..I...-I.EV.....I..V.S...KEWS.....L.E.KE...K...A...EELENA.TKHKDATV.TA					52%
Sweet potato	MAAATSS...	Q.....VDHWKEQF.K.V..KR..VV.....M...IL..I...-MAHV..V.....QA..AE.K.....V.F...KE...RM..AK...LQNC.TKHAAAVMTA					52%
Greater plantain	--MATSS...	Q.YSV.SVE..KD.LEKS..AK...V.....F...IL..L...-T.HVM.....AISVE.E.....V.L...KPI.RL..AK.E.LLA..TTHGTVVA					52%

Glutathione transferase ----MAGE---KGLVLLDWFVSPFGQVRVIALAEKGLPYEYAEEDLMA-GKSDRLLRANPVHKKIPVLLH---DGRPVNESLIILQYLEDAFPDA-PALLP---SDPYARAQARFWADYVDKKVYDCGSRLWKLKGE----PQAQARAEMLDILKTLDGA
Barley V...I...S.....N.D.....T...V.....E.....
Maize MQVA...T-K.....C.....IA...S.QE.LGGA...I...S.....C...V...E...E...E.S.R.....DTA.....A.S...EA.T...R.D---AR.....IVQV.RN...E
Oil palm ----.TAAEKE.V.....C.....VE...K.N.SD--.PL..KM..I.....I---H...IC...V.V..IDEVWS-NN.....E...K.....F.....E.T.....AHEE...KD.IE...L.E.E
Rice ----.G---E.....A.....C.....K...D.S.QE.LG-A...L...S...I.A.V...GDG...A.C...A.E.D...T.R...SAAD.....R.....PV.T...V...EGVRAA.G.G.LVEA.R...E
Soy ----.SD----EV.....P...M.....IK...K...RN--.PL..QM.....I---N.K.IC...AV...EV.N.RN.....Q...T.....I.....L.RKI.TS.....EKEA.KK.F.EA...L.EEQ
Papaya ----.D----EV.....P...M.I.....IH...K.N.RN--.PL..QM.....I---N.K.IC...QI...IDEVWS.KA.....Q.....M.EA.R.V.TT.....E.EG.KK.FIE...E.E
Mallow ----.T----EV.....P...M.S.....IN...K...RN--.PL..QM.....I---N.K.IC...QV...DEV.H.KS.....HQ.V...F...M.EQ...V...T.....E.T.KK.FIES...L.E.E
Vine ----.D----EII.....P.M...M.....K...R...WN--.PL..EM.....A.I---N.K.IC...V...DEVWCDKS.....Q.....I...L.ELGRKI.ST.....E.ET.KK.F.EC...L.E.E
Pea ----.N----EV.....P...M.L.L.....IK...K...RN--.PL..QM.....I---N...C...AV...DEV.S.RS.....Q.....I.EV.RNV.TK.....E.EA.KK.FI.A...L.EEQ
Ricinus ----.D----EVI...A...M.....VK...R.N.RN--.PL..EM.....I---N...IC...AV...DEV.H.ES.....S.P.H...F...I.EL.RKI.TT.....D.EAGKK.FI.A...L.E.E
Tobacco ----.D----EV...TY...M.VS.....IQ...K.Q...N--.TPL..QM..I.....I---N.K.IC...VE.IDEVWK.KS.-FM.....K.....I.ES.KKM.TS.V----D.EA.NK.FIEC...L.E.E
Camomile ----.ENT---NV...TT.A.G.M.Q.....IE...K...SN--.SL..EM.....C.N...D...S.KS.S.....L.....AAR...TT.....E.ET...K.F.W...V.E.Q
Chili ----.ND---EVI...P.M.M.L.....EVK...SK...RN--.PL..QM.....I---N.K.IC...I.AVE.IDEVWK.KA.....T...E.....I...FF.SSRKI.TT.....E.EA.KKDFIEC.V.E..
Thale cress ----.N----EVI.....P.M.RM.T...R...VEF...R...RN--.PL..QM..I.....I---N.K...I.QV...IDEVWSHKN.-I.....L.....FI...L..AQRKV.AT.....E.EAGKKDFIE...ESE

Glutathione transferase LGDKPFFGGDKFGFVDAAPFTAWFHSYERYGEFSLPEVAPKIAAWAKRCGERESVAKSLYSPDKVYDFIGLLKKKYGIE-
Barley A.....E.....R.... 95%
Maize ...A...EA...V.LV.VP.LP.....D.VA.I..RL...R.AQ...RTLHP.E.DE.N...T... 67%
Oil palm ...KY...ET...V.V...YT..TC.K.VD.EC..L...K...S.VHD.H...E.V.K...F.L... 65%
Rice ..E.E...E-...V.LV.MMP.VY.FA...G..VE.EC.RV...R..M..D...G..R..EEI.....R.H...DD 63%
Soy ...TY...NL...I.LV..YT..KA..TF.TLN.ESEC..FI.....LQK.....PDQQ...E.MD.R..L... 59%
Papaya ..E.Y...ES..Y..LT.I..YT..SV.SF.KM.IEAEC..LFS.V...L.K...S..PDQ...G.VLE.R.AL... 58%
Mallow ..ASLT...ENL.Y..GVLV..YS.VYA..KC.N.NIEPEC..LI.....M.K...PDQE...VLQ...IY... 58%
Vine ..E..Y...E.I...V.LVT.SC..YA..TF.N..IEAEC..LI..T...M.K...SS..ED.H..HG..MGMR.RF... 57%
Pea TY...E.L.Y..I.LI..YT..KA..VF.NLN.EKEC..FIT...MQI.N.SR..PDQH...E..VEIR.R... 56%
Ricinus Y...ES..Y..V.LI..YS..YA..TC.N...EAEC..FM..I...LQK...S.A.PDQQ...E.VLE...V... 56%
Tobacco Y...ER...M.LM.YYS..P...KF.N..IEAEC..VE...K.VQK...S...AD.....Y.VMA.Q.W..A- 55%
Camomile LI..S...A.I.LI...C..Y.L.TI.NM..EKEC..V.V...M...S...AD.H...E..LQ...L... 55%
Chili ..E.....N...V.LIG.YS..FA..TC.N...TEAEC..FVT...MQ.D...PDQH..LE.VQT.R..... 55%
Thale cress Y.S..D..Y..I.LIG.YT..PA..KFAN..IESEV..LI..V.K.LQ.....PD.E..TE.VSE.R..FVP-- 52%

1-Cys-peroxiredoxin MPG-LTIGDTPNLELDSTHGKIRIHGYVNGYVILFSGPGDFTPVCTTELAAMANYAKEFEKRGVKLLGISCDVQSHKEWTKDIEAYK-----PGSRVTYPIADPDRSAIKQLNMVDPDEKDGQGG-LPSRTLHIVGPDKVKLSFLYPSC
Barley K.....A.....K.....
Rye A.....K.....AE.....K.....
Rice F.DT.I.....G.....D.....D.I.....N.....S.E.....SN.GH...A.....K.....
Maize D.A.I...A.....M...G.....E.RQ...V...GGKQQQQATTTK..F..L...A.D..R.....AA.RSM...A..V....A.....AT
Sunflower NL...DSFT.I.....G...A..DK.AQ.....L.....I.....N-----K.KK...A...N.EI.....AS..N...A.....KI.....AS
Poplar S.....VET...V.KL...I-DTWT.....GK..AH.P..A.....L...S.A.V.....T-----CK...I...K.EL..I.....SS.HNV...A....A..RI.....AS
Ricinus L...I...VET...I.KL...I-DTWT.....GK..A..Q..ANK.....L...L.V..I.....T-----K...I...S.QL.H....A...DS.KNV...A.....KI.....AS
Thale cress I.L.....VET..D.FKL...FA.SWTV.....G...K..H..D.....L.....D.I.....FN-----H..K.N...I...NKEI.P...I..I.NG-----A.....SKI.....T
Barrel clover I.D..V.T.Q...KL.HFCSDSWT.....GK..Q..S..N...M...M...LE..K..I...HT-----AK.N...IS..K.EI.....SN..N...A.....KI.....AQ
Buckwheat SI...QVET...SFK...FI.DSW.....GK..K.EE..T.....L...IA..K..I..V..FT-----K.R...I...K.EV.TK.....SS.SQ...A.....K.....AT
Oil palm I.D.LV....P.Q...FI.D.WAFI...A.....GKI..L..E..D.....V..V..I.....T-----CN.R...V...EV.R.....Q..SS.LE...A..VI...RI...I...AT
Rape I.L.....VET..KNFKL...FADSWTV.....G..GK..H...Q.....L...I...D.IP...FT-----K...I...NKEI.P...I..I.NG-----A..V...CKI.....T
Bog ..G.GWAL...L..DIQA...M.H.KVR..CKD.WT.I.....YP.....GKI..A.NP.....L.T.T.ED.QG..I...S..T-----DAP.L...L.....KITVA...M.....AN.KP.A..A...I...CRL...L...GT
Zebra finch LL..EA.DFEA.T.Q.R..F..FL.DSWG.....R.....GRA.QL.P..S..N..MIAL.I.S..D.LS.C..VN..NGEQ-----AEKLPF..I..KN.ELAVK.G.L...L.KD.MP.TA.VVF.F...KL...I...AT

1-Cys-peroxiredoxin	TGRNMDEVVRAVDSLLTAAKH--KVATPANWKPGECCVVIAPGVSDEEAKMFPGGFETADLPSSKKGYLRFTKV--	
Barley	--	99%
Rye	L.....K.....	96%
Rice	V.....A.Q.....--A...V.....R...P....D...EK....D....G.....G-	86%
Maize	L.....GG.....A.....R.....	83%
Sunflower	L...IK.SQ.--I...V...E..P....S..ND..R...K..Q.V...N.D....S.--	74%
Poplar	VL...ERSS.N--I.....D...S.S.....L...K.VGI.SN.....N.DH	72%
Ricinus	M.V.E..QR.....I...D...DP...S.S..TD.....YK.V...E.....N.D-	72%
Thale cress	L..L...M.S..NN.I...V...DQP...S.A.....K.....R.E.S-	72%
Barrel clover	L.V.E..QK.S.Y--I.....P...S.D.T.DQ..E.....K.....E....N.--	71%
Buckwheat	E...V.E..QK..ND--VD.Q...DEA..S.S.....H.YR.V.....Q.--	71%
Oil palm	V.E..QKTS.L--I...V...K...S.S..N...E....YD.V...E....NI--	70%
Rape	L..L...M....KN.I...V...D.P...S.A.....L...K..K.....VAD.S-	70%
Bog	F...L.VL...QL.S.--I.....QK..P...S.S...K..Q....W..VN...--KA..M.F.D-	70%
Zebra finch	F..IL.VL...QLT.YK--....VD....DS.MVV.TLP.....L..K.VF.KE...G.K...Y.PQPE	58%

Dehydrin -----MADYG-GEYGHYP-----RVDEYGNPV-----PPVDQYGNPIPREPGQVPAYTSGGAAPPYSSDGAGAVTSADYGAGVTPGYGLSG--AVHPQESVVG-----AVFPSGTAHTHEGALSG-----
Barley -----A...G.A..VT-----A.E...K...G...H...P...A.YA...V...GLAP---GETTAYAYEGAVSG
Rice -----EHAT.V.....Q.....V.D.--A.RD.AA.YVA-----P.P-.VSTGD...A.AE.P..H.SA.MSGAAAA..A.G.E.Y.RD.GG-----
Oil palm -----MADPIRR-----T.....IPEHQGHGGAVT-----GGT...TTT-----TPHEGVLH.EG-----
Thale cress -----ES.Q-----NQSGAQQTH-----QQL..F...F-----AT.GA..TA-----APAVA..GG-----
Grass -----EYQ-----QQQHDQATT-----NR..E...VA-----H.VGTG-----MGAHGGVGTGAAA.G-----
Grass -----EYQG-----QQQHGGQAT-----NR..E...VA-----Q.VGT-----GAAA.G-----
Coffee -----Q..AEYGNQKSQ-----Y.....PVRQT.E...ARHG-----GTM.DY-----TTGTTGAY.GTT---AH
Ricinus -----EH.QNQYGAV..T-----E...MHHN-----GAA.TYTG-----ALE.GAGF.VG-----
Vine -----YQQDPCANPTRQ-----TGKT.G-----QT.....VHQT-----EAL.AY.A-----GTG-TGMH-----
Mexican sunflower ANYGGDKQYGRETRHTGDYGNPIHSA---TGGQ.DQEIRQTDEYGNPVRRT.E...VHSAT-----GGTM.DYGS-----T.LGQGTG.IGT.GYGTTGHQGLRTGLGHTTG--G
Rape -----LKDERGNPIHL-----T..H-----PVQLT.EF...MHITG-----VASSAPQYK-----ESVTGNIQEYRTAAP---PAG
Sallow thorn -----DEYGNPIHNGSTGINTTG.QQ-----QTAGLYGTGHGSG-----YGTG.TQFG-----TTQHTAGH.TG-----
Sunflower -----QYGRETRHTGDYENPIHS---TGGQ.DQD.RQTDEYGNPVRRT.E...VHSAT-----GGTM.DY.S-----T.LGQGTG.LGT---VGHTTGGTG
Green tea -----HNSNQYGNP.RQ-----T.....PRKTDEFGDPVRI.E...VHHT-----GTM.DY.T-----TTGTTGVH.THT.T-----TGTYGTGTT

Dehydrin SLAPGETTAYAYEGMVGSGIGT--G-----DQIQPTK-----EGHTTLGETLRRS--SSSSSSSSSEDDGQGGRRQKKKSMKAKIKEKLPGSHKQEEHKAG-----HTVPP-AGTGT-----HEK-----
Barley G...G.....AGA-----R...A-----E.....A...GS.....GI.E.....NQ.H.....AAA.A.....KGIME
Rice VVPPAGEKTF...T.SAAGV.GAS---G.L...TRE-----GK.....I.E.....Q.QA-----GHTA.A...GTGTH-----AAGK...KGIVE
Oil palm -----RQQ-----VH.G.EE-----HP.GRHH---G...GL.E.....GGHKS-----EEHQQTDEGQ...KGMME
Thale cress -----SGM.H---G.....L...R...GITE.....H.DSNKTSSL-----GSTTTAYD...-VH...KGMME
Grass -----HF...R-----EEHKA.GI.Q---G...M...-R...GI.D...G.GDQQQT...TYGQQGHTG-M...GNYGQP GHTGMA----GTDGTG..KGIMD
Grass -----HF...M-----QEHKA.GI.Q---G...M...-R...GI.D...G.DQQQT...TYGQQGHTG-T...GNYGQP GHTGMA----GTD---E.KGIMD
Coffee GTYATG..GTTGT.AYATQP..DV-----KEHHGL-----GM.H---G.G...-G.E.....GHK.AQP...QEYSSATAAPG-----YGGEGVQHE--KGIMD
Ricinus -----HHKEHHG-----ITGK.H---G.L...E.H...-GL.E.....HK.DR...SQ.TSTTTP.G-----YNSTGEHH.G.RGIID
Vine -----EH.....Q..HQQ-----PGV.N---G...-G.E...RI..M-GRKDEQ-----KQT-SATS.P-----GQGGQQ---KGMME
Mexican sunflower TDYTSGGRSTEQT.YQ.L.TESAF.GTTGTF.N..SATPVGGVGLSTGTGAGFR--TGIGTGV.H.--G.G...-GHSQEE-----QYQSQTIT.AGGVGRAG-----YGETHEM---KGMME
Rape VA.GTGVA.TTAA.VATGETT.G-----Q.HHES-----G.D...S.G-KHKDEQ-----TPSTATTTP.TT-----TGAAAADQHHEKKGILE
Sallow thorn -----TLT-----STA.AG-----HGQTK...-G.G...-GL.E.....GHKQHQHP-----GQYKSVTT.IITPDSGY--EQSGQQHQQQHDK-GIMD
Sunflower TDYTSGGRSTGQT.YQ.L.TESEF.GTTGTF.N..SATPIGGTGLSSGTGAGFGGI.TG.GTGI.H.--G.G...-GVMQ...-GHSQEE-----QYQSQTIT...YGETHEK---KGMME
Green tea GTYGTGMGTTGTT.TH.LST..GG-----HH.QHAD-----GV.H.--G...-GLTQ...-GHKDQT-----PQYGNTTT.PGAATTGGY---GYGGEDQQQYPEKKGMME

Dehydrin	-IKEKLPG-----	
Barley	K.....HH-----	76%
Rice	K.....HGHH-----	58%
Oil palm	K.....HH-----	54%
Thale cress	K.....GHH-----	47%
Grass	K.....QH-----	43%
Grass	K.....-----	43%
Coffee	K.....GHHN-----	42%
Ricinus	K...FT.GHHHHNEPRHSEHQNY	41%
Vine	K.....AH-----	40%
Mexican sunflower	K.....HH-----	37%
Rape	K.....HHNHHP-----	36%
Sallow thorn	K..D...SFSLI-----	36%
Sunflower	K.....HH-----	35%
Green tea	K.....HTTTNK-----	32%