

A molecular and proteomic investigation of proteins rapidly released from triticale pollen upon hydration

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Abstract Analysis of Triticale ($\times$ *Triticosecale* Wittmack cv. AC Alta) mature pollen proteins quickly released upon hydration was performed using two-dimensional gel electrophoresis followed by mass spectrometry. A total of 17 distinct protein families were identified and these included expansins, profilins, and various enzymes, many of which are pollen allergens. The corresponding genes were obtained and expression studies revealed that the majority of these genes were only expressed in developing anthers and pollen. Some genes including glucanase, glutathione peroxidase, glutaredoxin, and a profilin were found to be widely expressed in different reproductive and vegetative tissues. Group 11 pollen allergens, polygalacturonase, and actin depolymerizing factor were characterized for the first time in the Triticeae. This study represents a distinctive combination of proteomic and molecular analyses of the major cereal pollen proteins released upon hydration and therefore at the forefront of pollen-stigma interactions.

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Introduction

Triticale ($\times$ *Triticosecale* Wittmack) is an intergeneric hybrid between wheat (*Triticum* spp.) and rye (*Secale* spp.) which combines high yield potential and good adaptation to environmental stresses (Oettler 2005). Triticale can be genetically modified for the production of various biopolymers (e.g. bioplastics, BioSteel), adhesives, industrial enzymes (e.g. cellulase, glucanase), and by-products such as ethanol and high-strength fibres (Gressel 2008). Although triticale is a self-pollinated species, there is a small risk of cross-pollination under specific conditions (Kavanagh et al. 2010) and the adoption of genetically modified triticale as a bio-industrial platform may require mechanisms to prevent gene flow. As one of the first steps in developing such mechanisms, we have characterized the major proteins rapidly released upon pollen hydration as they are likely to be at the forefront of pollen/stigma interactions.

Upon maturation, the anther dehisces and releases dehydrated metabolically inert pollen grains. When the pollen grain lands on the stigma, it hydrates, germinates, and produces a pollen tube which penetrates the stigma wall and grows to reach the embryo sac where it releases two sperm cells enabling double fertilization (Wang et al. 2010). The grass pollen cell wall can be divided into an inner intine layer and an outer exine layer covered by the pollen coat. Pollen-specific proteins including expansins, profilins, and actin depolymerizing factors (Valdivia et al. 2009), as well as enzymes such as polygalacturonase (Kim

et al. 2006), glutathione S-transferase (Zhu et al. 2010), and glucanase (Suen et al. 2003) have been identified as important constituents of the grass pollen wall. These proteins are believed to play important roles in pollen-pistil interactions, adhesion, germination, and pollen tube growth leading to successful fertilization (Heslop-Harrison 1987; Grote et al. 2008) or in providing protection against pathogen attack (Andersson and Lidholm 2003). Many pollen-specific proteins have also been identified as pollen allergens and classified into 11 groups based on their IgE response in humans (Radauer and Breiteneder 2006; Hrabina et al. 2008), however, their role in pollen development or function often remains poorly understood.

Many of the pollen proteins rapidly released upon hydration are believed to be stored in the cell wall, nevertheless, few studies have attempted to characterize the grass pollen cell wall proteome (Radlowski et al. 1996; Kalinowski et al. 2002, 2005, 2006). During development, pollen wall proteins originate either from the pollen cytoplasm itself or from the surrounding tapetum which lines the inside of the anther locule. The proteins present in the cereal pollen cell wall are believed to be easily released in liquid medium (Heslop-Harrison 1987; Wang et al. 2006). Different approaches have been reported to isolate grass pollen coat/cell wall proteins without contaminating proteins from the pollen cytoplasm (Kalinowski et al. 2002; Grote et al. 2005; Wang et al. 2006). In these protocols, the purity of the pollen cell wall proteins was assured by verifying the integrity of the pollen. Thus, provided the pollen remains intact, quick exposure of mature grass pollen to an aqueous medium may mimic in vitro the rapid release of proteins upon pollen hydration on the stigma papillae. The two-dimensional gel electrophoresis (2-DE) profile of rapidly released pollen coat proteins from *S. cereale*, a cross-fertilizing grass species, has been previously reported (Kalinowski et al. 2002), however these proteins were not identified or characterized. In the present study, we optimized the protocol for the isolation of pollen cell wall/coat proteins from triticale. The major rapidly released proteins and their corresponding genes were characterized in detail. This report not only confirms existing data on grass pollen proteins, but considerably expands our knowledge of Triticeae proteins released immediately upon pollen hydration.

Materials and methods

Plant materials and growth conditions

Triticale ($\times$ *Triticosecale* Witt. cv. AC Alta) plants were grown from seed in 15 cm diameter plastic pots containing soil:peat:vermiculite (3:1:1). Plants were maintained in

controlled growth chambers at 20°C day temperature under 16 h light (250–275 $\mu\text{E m}^{-2} \text{s}^{-1}$) composed of fluorescent and incandescent lights. The night temperature was 18°C and the relative humidity was constant at 70%. Plants were fertilized weekly with a commercial 20:20:20 fertilizer solution and watered twice daily. Flowers were produced after 2–3 months under these growth conditions. To collect mature pollen, the florets with protruding yellow anthers were harvested and the longitudinal groove of the anthers was disrupted with the tip of sharp forceps to release the mature pollen onto wax paper. The purity of isolated pollen was verified by light microscopy. For developmental expression studies, anthers of 1.5, 2.5, 3.5, 4.5, and 5.5 mm in length were removed from flowers at different growth stages under the dissecting microscope. These stages mostly correspond to tetrad (1.5 mm), uninucleate microspore (2.5 mm), bicellular pollen (4.5 mm), and tricellular pollen (5.5 mm) (Tran 2011). Anther- or pistil-removed florets, coleoptiles, developing seeds at the milk stage, roots, stigmas, and ovaries were also collected. All tissues were flash-frozen in liquid nitrogen and stored at -80°C for later use.

Protein extraction, electrophoresis and mass spectrometry

Various protein extraction buffers and protocols were evaluated for their capacity to enrich for rapidly released pollen proteins or to extract total pollen proteins from fresh or frozen triticale pollen. Most buffers tested were Tris/EDTA-based, contained Complete Mini Protease Inhibitor Cocktail-EDTA-free (Roche, Laval QC) with or without the addition of dithiothreitol (DTT), CHAPS, and sucrose (5–15% w/v). Distilled water containing protease inhibitors was also used with limited results (results not shown).

The extraction method found most suitable for the enrichment of rapidly released pollen proteins was the following. The extraction buffer comprised 20 mM Tris (pH 7.5), 0.5 mM EDTA, 52 mM DTT, 32 mM CHAPS, and Complete Protease Inhibitor Mini Cocktail (as recommended by the manufacturer). Rapidly released pollen proteins were harvested from 10 s extractions at room temperature. Longer extractions resulted in increased levels of pollen cytoplasmic proteins (Fig. 1). Extractions were typically performed on 10–100 mg of whole triticale pollen (fresh or frozen) added to 150 μl of extraction buffer within a 1.5 ml centrifuge tube with gentle mixing. The tubes were then centrifuged at $21,000\times g$ for 1 min and the supernatants transferred to a clean tube. The supernatant was then clarified by a second round of centrifugation at $21,000\times g$ for 2 min and transferred to a fresh tube where the extracted proteins were precipitated in 10 volumes of ice cold acetone for 1–16 h at -20°C . After removal of the

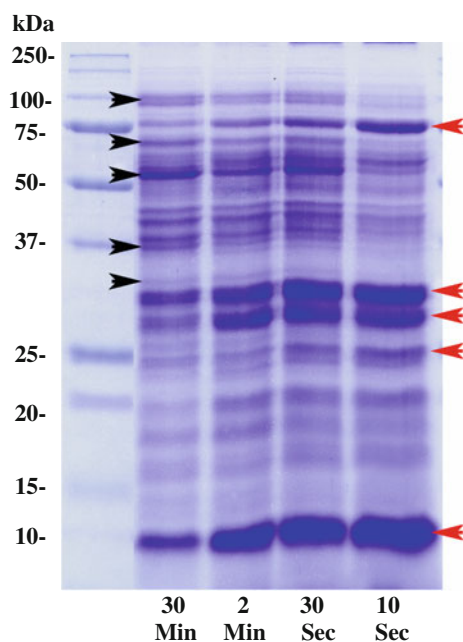


Fig. 1 1-D SDS-PAGE of different aqueous extractions of triticales pollen proteins. Different pollen samples were incubated in extraction buffer for the times indicated. *Red arrowheads* designate abundant proteins that are extracted quickly from the pollen, while *black arrowheads* show proteins that are enriched during longer extraction times. Molecular weight standards are shown in the right lane in kDa

acetone, air dried protein pellets were re-suspended in SDS-PAGE sample buffer or isoelectric focusing buffer for 1-D or 2-DE, respectively. Protein concentration measurements were performed with the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions.

Protein electrophoresis (1-D and 2-D gels) was performed as described by Gagnon et al. (2010). In-gel trypsin digestion of abundant protein spots was performed according to Kinter and Sherman (2000) and tryptic peptides were eluted from gel plugs into 100 mM sodium carbonate (pH 10). Peptide desalting and mass spectrometry were carried out at Protana, Toronto, ON, Canada (<http://www.protana.com>). Peptides were resolved by liquid chromatography then introduced into a MDS-SCIEX QSTAR XL mass spectrometer by nanospray ionization (LC-Nanospray-Quadrupole-TOF). Mass spectra were analyzed with PEAKS 4.2 software (<http://www.bioinformaticsolutions.com>) and peptide amino acid sequences were determined manually allowing for carbamido-methylation of cysteine and oxidation of methionine residues (Kinter and Sherman 2000). Peptides sequences were aligned with public protein sequences using BLASTp2 (<http://dove.embl-heidelberg.de/Blast2/msblast.html>) and protein matches involved at least 2 peptides (except for 3 samples discussed below) and met a minimum 95% probability cut-off value.

Total RNA preparation

Frozen tissues were ground to a fine powder in liquid nitrogen and total RNA was extracted using the Concert Plant RNA Reagent (Invitrogen, Burlington, ON, Canada) following the manufacturer's instructions. RNA was re-suspended in nuclease-free water and the concentration was measured using the ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA).

Rapid amplification of cDNA Ends (RACE) and DNA sequencing

RACE was performed using the First Choice RLM-RACE Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. A combination of 3' RACE and 5' RACE was performed to obtain cDNA clones containing full length coding sequences. Equal amounts of total RNA from 1.5 to 5.5 mm anthers were pooled and 1 µg of this pooled RNA was used to prepare first strand cDNA. For nested PCR, either the 3' or the 5' RACE inner primer was used with a gene-specific primer. Gene-specific primers were derived from public sequences matching the MS peptide sequences, preferably, those from Triticeae. A list of all the gene-specific primers used in this study can be found in Table S1 of the Supplemental Data. All PCR amplifications were carried out using AccuPrimeTaq polymerase (Invitrogen, Burlington, ON, Canada) with the following conditions: 1 cycle at 95°C for 3 min, 35 cycles at 94°C for 30 s, 60°C for 30 s to 2 min, and 68°C for 45 s. A final extension of 7 min at 68°C was also performed. All triticales cDNA sequences submitted to GenBank were the result of a single PCR product using primers derived from RACE experiments. The amplified products were purified using the Qiaquick gel extraction kit (Qiagen, Mississauga, ON, Canada), cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), and sequenced (DNA Sequencing and Synthesis Facility, Ottawa Health Research Institute, Ottawa, ON, Canada).

Sequence analysis

Homology searches of cloned cDNA sequences and their deduced amino acid sequences were performed using BLAST (GenBank <http://www.ncbi.nlm.nih.gov/blast>). Multiple sequence alignments were carried out using CLUSTALW (DNASTAR Lasergene). Online tools were used for open reading frame and deduced amino acid sequence searches (<http://www.bioinformatics.org/sms/>), for isoelectric point, molecular weight, and protein domain searches (<http://ca.expasy.org/>), (<http://hydra.icgeb.trieste.it/servers/protein/sbase/>), and for potential glycosylation sites and signal sequences (<http://www.cbs.dtu.dk/index>).

[shtml](#)). Table S2 lists the cDNA clones obtained with their corresponding GenBank accession numbers, whereas Table S3 shows the few examples where the deduced protein sequence obtained from the cDNA varied from the peptide sequence obtained by MS analysis.

Northern blot analysis

Northern blot analysis was performed as described by Wu et al. (2008). Two micrograms of total RNA isolated from different plant tissues were loaded in each lane except for the total RNA sample from mature pollen, this sample was restricted to 100 ng due to limited tissue availability and also because preliminary experiments indicated that two micrograms of total pollen RNA often gave hybridization signals that were relatively too strong (results not shown). The probes for hybridization were prepared from the representative cDNA clones by convenient restriction enzyme digestion (Table S4) and were ^{32}P -labelled by random priming with the DecaLabel DNA Labelling kit (MBI Fermentas, Burlington, ON, Canada). Hybridizations were performed with Church's buffer at 65°C (Wu et al. 2008) and the membranes were washed and then analyzed with a STORM 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

Results and discussion

Pollen grains of cereals (including triticale) can have thin walls which render them unstable in aqueous buffer (Grote et al. 2001). Upon hydration, these thin-walled pollen grains burst, rapidly expelling their cytoplasmic contents through the germination pore. A number of different approaches were evaluated in order to extract rapidly released pollen proteins with minimal contamination by cytoplasmic proteins. These included various isotonic solutions, mechanical extraction methods, and extraction times (results not shown). Enrichment of pollen wall proteins versus total pollen proteins was found to be primarily a function of time in which whole pollen (fresh or frozen) was in contact with the extraction buffer. Pollen proteins released upon hydration were enriched during very short extraction periods, less than 30 s in duration. During longer extraction periods, pollen swelling would occur rapidly and interior protein containing particles were released into the extraction buffer through the pore. In some cases, pollen lysis was also evident, resulting in the release of total cytosolic contents into the extraction buffer. Triticale pollen proteins obtained with longer extraction times are shown in Fig. 1.

The method consisting of a 10 s extraction in an aqueous buffer solution was found to be the most reliable. With this procedure, more than 95% of pollen grains remained intact (based on microscopic observations), thus

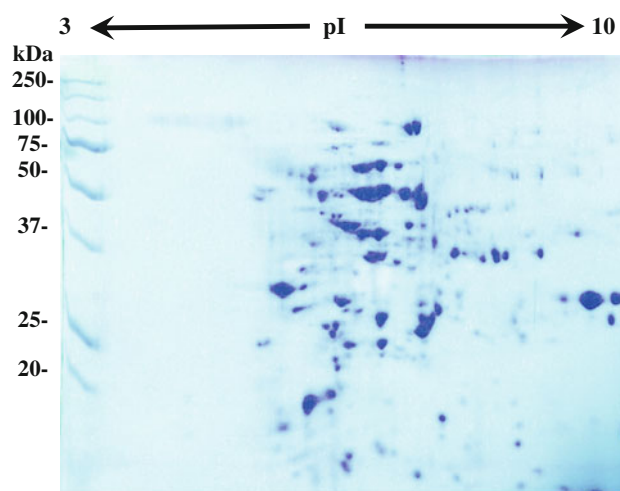


Fig. 2 2-D PAGE of triticale pollen proteins released after a 40 min extraction. Molecular weight standards are shown in the right lane in kDa

minimizing the contamination by cytoplasmic proteins as can be seen by comparing Figs. 2 and 3. Previous reports have also indicated that pollen coat proteins from various grasses can be selectively extracted (Radlowski et al. 1996; Kalinowski and Wojciechowska 2003; Kalinowski et al. 2002, 2005, 2006). The 2-DE profiles of pollen coat and pollen protoplast proteins of rye (*Secale cereale*), a member of the Triticeae and a contributor to the triticale genome, have previously been reported by Kalinowski et al. (2002) although proteins were not identified. Their extraction method was based on obtaining 10 sequential fractions of pollen coat proteins after 10 or 15 min incubations and the majority of the proteins which were purified were common to both the pollen coat fraction and the pollen protoplast.

Numerous triticale pollen proteins rapidly released upon hydration were separated by 2-DE and the 30 most abundant protein spots were selected for MS characterization of which 22 (Fig. 3) yielded the peptides listed in Table 1. Occasionally we found multiple spots corresponding to the same protein as has been reported in other proteomic studies (Xu et al. 2010). For example, the group 5 pollen allergen was identified at three locations (spots 4, 5, and 22). The presence of multiple spots for the same protein, as well as discrepancies between the observed and predicted molecular weight and pI of certain proteins, can be due to a number of factors including post-translational modifications, protein degradation, partial synthesis of proteins during pollen maturation, or protein translation from alternatively spliced mRNAs.

The cDNA clones comprising full-length coding sequences were obtained using the MS-derived peptide sequences, with the exception of the group 5 pollen

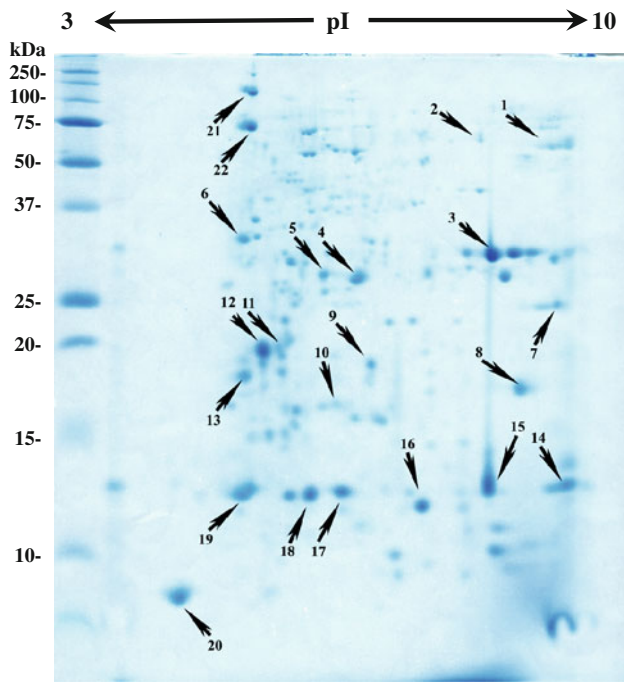


Fig. 3 2-D PAGE of tritcale pollen proteins released quickly upon hydration. Numbered spots yielded good MS/MS peptide sequencing data and were assigned the following putative identifications: Spot 1—Group 4 pollen allergen; Spot 2—Polygalacturonase; Spot 3—Expansin B4; Spot 4—Group 5 pollen allergen; Spot 5—Group 5 pollen allergen; Spot 6—Endo- β -1,3-glucanase; Spot 7—Expansin B1; Spot 8—Cyclophilin; Spot 9—Glutathione peroxidase; Spot 10—Actin depolymerizing factor; Spot 11—Group 11 pollen allergen; Spot 12—Group 11 pollen allergen; Spot 13—Pollen-specific protein; Spot 14—Group 3 pollen allergen; Spot 15—Group 3 pollen allergen; Spot 16—Glutaredoxin; Spot 17—Group 2 pollen allergen; Spot 18—Group 2 pollen allergen; Spot 19—Pollen profilin; Spot 20—Group 7 pollen allergen; Spot 21—Profilin; Spot 22—Group 5 pollen allergen. A number of spots did not yield useful MS data (data not shown). Molecular weight standards are shown in kDa

allergen which could not be isolated. In cases where more than one cDNA sequence was obtained, the longest cDNA was chosen for further sequence analysis (Table 2). Thirteen gene families expressed specifically in the anther/pollen, while four were found to be expressed in reproductive as well as in vegetative tissues (Fig. 4) as described in detail below. A similar finding was observed by Kalinowski and Wojciechowska (2003), as they found 14.2% of all pollen peptides and 25.7% of leaf peptides were shared in 2-DE analyses of *Aegilops geniculata* $\times$ *S. cereale* hybrids. The deduced protein from most representative cDNA clones faithfully displayed the MS peptides obtained, although a few mismatches were found in some cases (Table S3). Since tritcale is a hexaploid species harbouring the *Secale* R and *Triticum* A and D genomes, variant forms of a specific protein might have originated from polyploidization (Ma et al. 2004). Features of the deduced proteins such as known domains, potential

N-glycosylation sites and presence of a putative signal peptide are also summarized in Table 2. In the present study, 17 different protein groups were successfully analyzed by MS analysis and these are described below.

Expansin B1

Spot 7: Four different peptides were identified by MS analysis (Table 1) and the best match was to the expansin B1 protein from *Triticum aestivum* (20.4% coverage). The multigene expansin family can be divided in two main classes, α -expansins and β -expansins, which display only about 20% amino acid identity to each other, however, they are comparable in size, they share a number of conserved motifs, and they have similar wall-loosening activities (Cosgrove et al. 1997). In wheat, 10 sub-families of β -expansins, expansins B1-B10, have been characterized (Lin et al. 2005). Grass pollen β -expansins belong to the group 1 pollen allergens which show some homology with members of the group 2 and 3 pollen allergens (Petersen et al. 2006; Breen et al. 2010).

Two cDNA clones containing complete tritcale expansin B1 coding sequences including the observed MS peptides were obtained and called *ExM1* (Acc. No. JN578727) and *ExM2* (Acc. No. JF811348). These two cDNA clones showed 99.7% sequence identity and *ExM2* had high homology with expansin B1 from *T. aestivum* and several other cereal expansins (Table S5). Northern blot analysis was performed to ascertain the tissue-specificity and developmental expression pattern of expansin B1 in tritcale. The tritcale *ExM2* probe hybridized with a 1 kb transcript first detected in 3.5 mm anthers whose expression peaked in 4.5 mm anthers and persisted in 5.5 mm anthers. No signal was observed in other tissues (Fig. 4). Interestingly, no hybridization signal was observed in mature pollen (note that the mature pollen sample contains 20 $\times$ less RNA). Jin et al. (2006) have shown that the wheat *TaEXPB1* and *TaEXPB2* genes are also specifically expressed in the developing pollen, but not in mature pollen. Our results confirm their observations and further demonstrate that despite the absence of detectable mRNA, the β -expansin protein is present in mature pollen. Faint hybridizing bands of larger molecular weight could be observed in other tissues (Fig. 4) and may correspond to alternatively spliced transcripts or related expansin genes expressed in other wheat tissues (Lin et al. 2005).

The deduced proteins from cDNA clones *ExM1* and *ExM2* showed only one amino acid difference with each other and revealed a high degree of sequence identity with expansin B1 from *T. aestivum*, as well as homology with other expansins from *H. vulgare* and *O. sativa* (Table S5). *ExM2* displayed a predicted signal peptide, as well as a putative N-glycosylation

Table 1 Identification of major triticale pollen proteins quickly released upon hydration

Spot No.	Protein	MS peptides	Best protein match (gi)
1	Group 4 pollen allergen	SEKPETFVAVDLNK TAWVESGAQLGELYAIAK KYGIAAENVIDVK SSMSPDHFVAVR LLPVPTVTVFK TVQEGAVDLVNK	<i>T. aestivum</i> (55859458)
2	Polygalacturonase	AWASACGGTGK	<i>Z. mays</i> (89892725)
3	Expansin B4	LIGPFSVR TVVDDVIPK GCGSCFELK GWKPDTSYEAK SSNENYLALVIK TTWYGKPTGAGPK FLQGDGDVVGVDIK EVDKAPFHGMTSCGNIPIFK	<i>T. aestivum</i> (44894802)
4	Group 5 pollen allergen	KAIDQVDAAFRT KLSSSLELSYKL KFTVFQTALSKA KQAYAATVAAAPEVK KYDAYVATLTESLR	<i>H. vulgare</i> (1808987)
5	Group 5 pollen allergen	KAQGATPEAKY KFIPALEAAVKQ KAIDQVDAAFRT KLSSSLELSYKL KFTVFQTALSKA KQAYAATVAAAPEVK	<i>H. vulgare</i> (1808987)
6	Endo- β -1,3-glucanase	KATGAPLLANVYPYFAKR	<i>T. aestivum</i> (115305907)
7	Expansin B1	KDVIPANWKA RSFGELELQFRR KGSNPNYLAVLVKF KFVSDDGDDVQMDIQESK	<i>T. aestivum</i> (42601005)
8	Cyclophilin	TVENFR ALCTGEK IVMELYK QVVIADCGQL GNGTGGESIYGEK VFFDMTVGGAPAGR IVMELYKDAVPR HVVFGVEVVEGMDVV	<i>T. aestivum</i> (13925734)
9	Glutathione peroxidase	KGGLFGDSIKW RYAPTTSPLSIEKD KVDVNGNNVSPLYKF KVLIVNVASQCGLTNSNYTELSQLYPKY	<i>T. monococcum</i> (34334012)
10	Actin depolymerizing factor	KMLYASSKD KVQQVVVDR KIFFISWAPDTSRV MSNSASGMAVCDECK	<i>O. sativa</i> (50251759)
11	Group 11 pollen allergen	ECGGILQAYDL SPDASCAEIEEFR VEGATVAMDCR VPLTSNDGMK	<i>P. pratense</i> (47606039)

Table 1 continued

Spot No.	Protein	MS peptides	Best protein match (gi)
12	Group 11 pollen allergen	EGATVAMDCR VFDDVVPTDFK VPLTSNDGMK ECGGLQAYDLK SPDASCAELEEFR	<i>L. perenne</i> (47605808)
13	Pollen-specific allergen	IDGVTDETGYK DLPDYIVQGR ANPLGYLK VGISDNIR	<i>O. sativa</i> (20310)
14	Group 3 pollen allergen	IDYTRPNDLSEVELR GSEEWQPLTKKGDVWEVSCSKPLVGPFNFR FDEVFSTDFK	<i>T. aestivum</i> (972512)
15	Group 3 pollen allergen	EWQPLTK VWEVSCSK DSLSEVELR VFDEVFSTDFK	<i>T. aestivum</i> (972512)
16	Glutaredoxin	SYCPFCVQVK LLTQLGASFK TVPNVFINGK LVALLTEAGAISGSSSK	<i>T. aestivum</i> (32401362)
17	Group 2 pollen allergen	NVFDDVVPTDFK PPVSFTVEK EEWLPLKK VGTTY	<i>L. perenne</i> (126386)
18	Group 2 pollen allergen	VGTTYKP VFDDVLPTDFK	<i>L. perenne</i> (126386)
19	Pollen profilin	KGAGGITIKK RLGDYLVEQGM KYMVIQGEPEGAVIR	<i>P. pratense</i> (2415700)
20	Group 7 pollen allergen	TLGSTSADEVQR	<i>P. pratense</i> (14423846)
21	Profilin	LGDYLVEQGF YMVIQGEPEGVVIR	<i>H. vulgare</i> (326501768)
22	Group 5 pollen allergen	RAIADVFKG KGSNASTFTAKI RTIAAMCVAAKG RTIAAMCVAAKGM KFVPALESVKK KFTVFEEALTRT KFVPALESVKKK KFTVFESAFDKA KAAVAAAAVVPGPDKY KAYAATVAEAEVKF RIAATAADAAPVNDKF KFTDTYIPDVDR KLAYDSADGATPEAKY KKAYAATVAEAEVKF KKFTDTYIPDVDR KYDSFIILSESLRI KAAVAAAAVVPGPDKYKK KGPIAAEMQAVNQIDTAFR KGPIAAEMQAVNQIDTAFRIM RIAATAADAAPVNDKFTVFESAFDKA	<i>T. aestivum</i> (40644796)

Table 2 Characterization of representative triticales cDNA clones

Spot No.	cDNA clone	Accession No.	Protein	Length (a.a.)	Signal peptide (position)	Predicted pI/MW	Observed pI/MW	N-Glyc (position)	Domain/motif, position
1	<i>TriA4F3R4-1</i>	JN388239	Group 4 pollen allergen	520	Yes (22–23)	8.7/57.68	9/70	Yes (351)	FAD/FMN-containing dehydrogenase, S69-L516; FAD-binding domain, P75-D214;
2	<i>PGR2-7</i>	JN578712	Polygalacturonase	415	Yes (22–23)	7.0/41.45	7/72	Yes (93,224, 249,391)	Berberine (BBE)-like domain, A455-P514 Glycosyl hydrolase family-28, C72-A404
3	<i>ExpB4F5R3-2</i>	JF811349	Expansin B4	270	Yes (24–25)	7.9/29.16	8/31	Yes (33)	β -expansin Rare lipoprotein A (RlpA)-like double-psi beta-barrel, K82-F161;
4	<i>Tris5R8-5^a</i>	JN578726	Group 5 pollen allergen	243	No	NA	6/29	No	Family-45 endoglucanase-like domain, G65-G171; Cellulose-binding-like domain, N185-S266
5	<i>Tris5R8-5^a</i>	JN578726	Group 5 pollen allergen			NA	<6/29	No	Group 5 pollen allergen ribonuclease, M1-A222; Group 2 allergen superfamily, M1-A111
6	<i>GlucanR3-4</i>	JF811350	Endo- β -1,3-Glucanase	336	Yes (31–32)	4.8/32.15	9/25	No	Glycosyl hydrolase family-17, I32-F334
7	<i>ExM2</i>	JF811348	Expansin B1	265	Yes (22–23)	8.8/28.58	9/18	Yes (31)	β -expansin Rare lipoprotein A (RlpA)-like double-psi beta-barrel, I77-G150;
8	<i>CycloF4R-1</i>	JF811347	Cyclophilin	171	No	8.2/18.38	8/29	Yes (78,115)	Family-45 endoglucanase-like domain, G60-G166; Group 1 pollen allergen, I169-P250;
9	<i>Glutpx2</i>	JN578723	Glutathione peroxidase	168	No	7.2/18.45	6/18	Yes (48,132)	Cellulose-binding-like domain, N180-S261 Cyclophilin-type peptidyl-prolyl cis-trans isomerase, F7-Q170
10	<i>ADF4-5</i>	JF811346	Actin Depolymerizing Factor	139	No	5.7/16.00	6/17	No	Glutathione peroxidase, S9-K118 Actin-binding domain, D12-L139
11	<i>Tris11F8-9</i>	JN578707	Group 11 pollen allergen	167	Yes (22–23)	5.1/18.14	5/20	Yes (48)	Pollen Ole e 1 allergen/extensin domain, V32-C151
12	<i>Tris11F8-9</i>	JN578707	Group 11 pollen allergen			5.1/18.14	5/19	Yes (48)	
13	<i>PolSpecF8-5</i>	JN578710	Pollen-specific allergen	165	No	5.0/18.28	5/18	Yes (48,158)	Pollen Ole e 1 allergen/extensin domain, V32-C121
14	<i>TrisA3-1</i>	JN388244	Group 3 pollen allergen	118	Yes (22–23)	9.2/13.22	9/13	Yes (48)	Pollen allergen/expansin, K26-S105
15	<i>TrisA3-1</i>	JN388244	Group 3 pollen allergen			9.2/13.22	8/13		

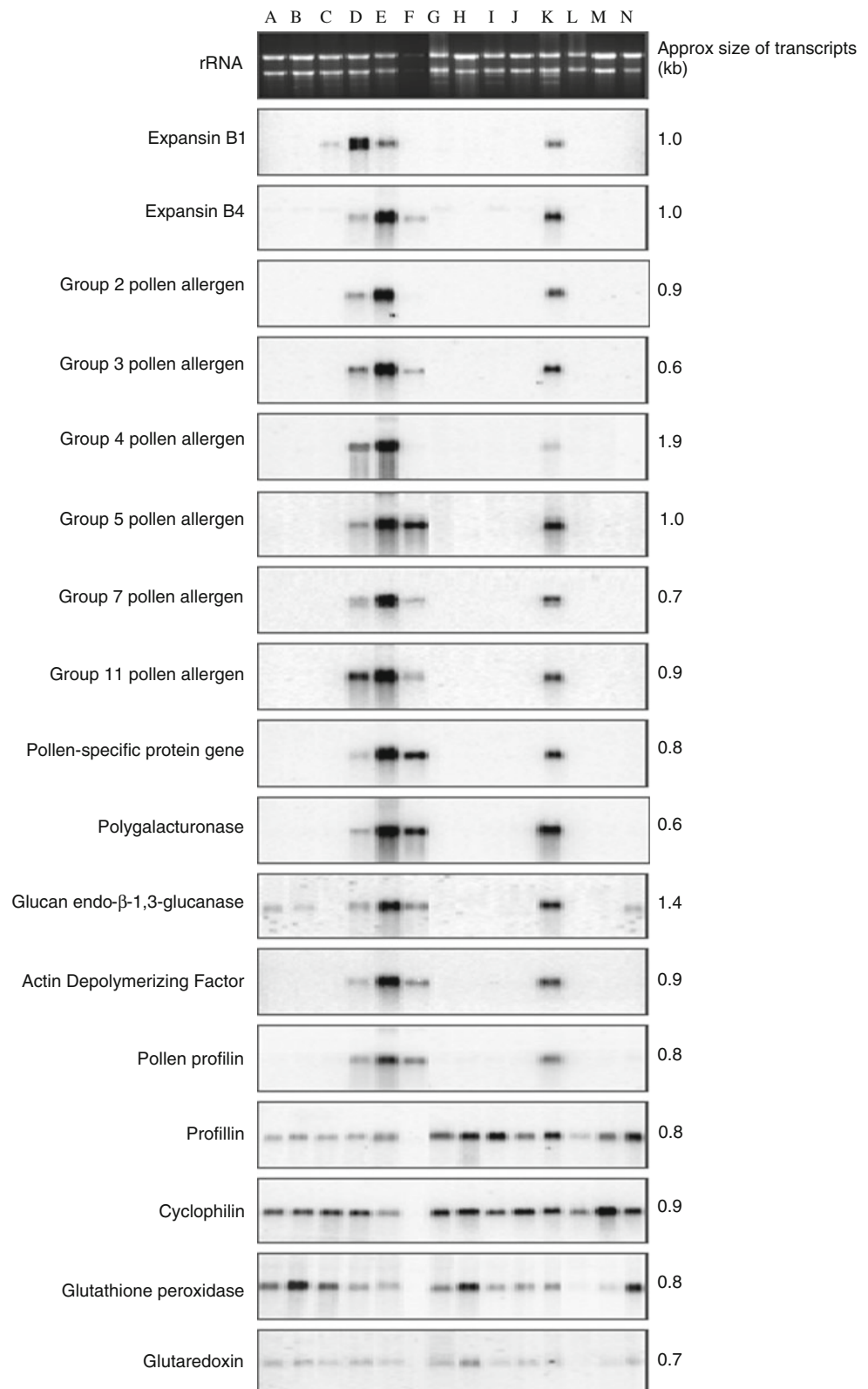
Table 2 continued

Spot No.	cDNA clone	Accession No.	Protein	Length (a.a.)	Signal peptide (position)	Predicted pI/MW	Observed pI/MW	N-Glyc (position)	Domain/motif, position
16	<i>Glut4H2</i>	JF811351	Glutaredoxin	131	No	6.0/11.65	7/12	No	Glutaredoxin, P32-L114; Glutathione (GSH) binding sites, K38, C41, C44, R83-P86, G97-D100; Thioltransferase activity site, C41-C44
17	<i>Polag2R4H-2</i>	JN388243	Group 2 pollen allergen	120	Yes (22–23)	8.0/13.35	6/12	No	Group 1 pollen allergen superfamily/ α -expansin, T30-T113
18	<i>Polag2R4H-2</i>	JN388243	Group 2 pollen allergen			8.0/13.35	<6/12	No	
19	<i>Pro3R4-2</i>	JN578720	Pollen profilin	131	No	5.0/14.02	5/12	No	Profilin, M2-M131; Poly-proline binding sites, W3, Y6, T31, W33, Y125, M131; Actin interaction sites, M1, I75-Q76, E78, K86, G91, T111-G113, M117, R121; PIP2-interaction sites, K71, K86
20	<i>G7F2-3</i>	JN388248	Group 7 pollen allergen	78	No	4.4/8.65	5/<10	No	Calcium-binding EF-hand α -helices, D4-L32 and E39-N67
21	<i>Pro3R2-2</i>	JN578721	Profilin	131	Yes (34–35)	4.9/14.18	5/12	No	Profilin, M2-F131; Poly-proline binding sites, W3, Y6, W33, Y125, F131; Actin interaction sites, A61, I75-Q76, E78, K86, G91, T111-G113, L117, R121; PIP2-interaction sites, K71, K86
22	<i>Tris5R8-5^a</i>	JN578726	Group 5 pollen allergen			NA	5/74	No	

a.a.: amino acid; N-Glyc: N-glycosylation site; NA: full length coding sequence not available

^a Partial coding sequence

Fig. 4 Northern blot analysis of transcripts corresponding to triticale pollen proteins identified by MS. (A) 1.5 mm (tetrad), (B) 2.5 mm (uninucleate microspore), (C) 3.5 mm, (D) 4.5 mm (bicellular pollen), and (E) 5.5 mm (tricellular pollen) anthers, (F) mature pollen, (G) flowers with anthers removed, (H) roots, (I) coleoptiles, (J) developing seeds, (K) flowers with pistils removed, (L) pistils, (M) ovaries, and (N) stigmas. Approximately 2 μ g of total RNA was loaded in each lane except for lane F—mature pollen (100 ng). The approximate size of the detected transcripts is indicated



site at position N31 characteristic of β -expansins (Li et al. 2003). The typical β -expansin domains, the group 1 pollen allergen motif, a rare lipoprotein A (Rlp-A)-like double-psi

beta-barrel motif, the family-45 endoglucanase-like domain, and the cellulose-binding-like domain (CBD) were found in ExM2 (Table 2).

The RlpA-like domain is found in the N-terminal region of group 1 pollen allergens from *Phleum pratense*, *T. aestivum*, and *O. sativa* (Jiang et al. 2005; Russell et al. 2008; Ivanciuc et al. 2009; Breen et al. 2010) although its function is not well understood. The N-terminal endoglucanase domain is believed responsible for the hydrolysis of the H-bonds between cell wall polysaccharides (e.g., hemicelluloses and cellulose), whereas the C-terminal CBD mediates the binding of expansin to cellulose microfibrils (Barre and Rougé 2002). The β -expansins are thought to loosen the cell walls of the stigma and style to facilitate the penetration of the pollen tube. In studies lowering the expression of maize pollen expansin B1, Valdivia et al. (2009) have shown that it is critical for proper pollen maturation and the initial penetration of the silk. Signal peptides at the N-terminus of β -expansins target these proteins to the pollen surface to loosen the stigma wall for pollen tube penetration (Jin et al. 2006). For example, expansin B1 produced inside the rye grass pollen was shown to move to the pollen surface through the cell wall when the pollen is moistened (Valdivia et al. 2009). Both the location and putative role of pollen β -expansins support the need for their rapid release upon hydration and landing on the triticale stigma.

Expansin B4

Spot 3: Eight different peptides were identified by MS analysis (Table 1) and the best match was the expansin B4 protein from *T. aestivum* (35% coverage). Like expansin B1, expansin B4 is a member of β -expansins containing the group 1 pollen allergen domain, although the two wheat expansins TaEXPB1 and TaEXPB4 show only 28% identity to each other (Cosgrove et al. 1997; Lin et al. 2005). Three triticale cDNA clones containing a complete expansin B4 coding sequence were obtained and identified as *ExpB4F5-1* (Acc. No. JN578728), *ExpB4F5-2* (Acc. No. JN578729), and *ExpB4F5R3-2* (Acc. No. JF811349). These clones showed greater than 97% sequence identity to each other and the longest clone, *ExpB4F5R3-2*, was selected for further characterization. As expected, it had very high sequence identity with expansin B4 from *T. aestivum*, in addition, considerable sequence identity was observed with group 1 pollen allergens from other grasses (Table S5). Northern blot analysis indicated that the highest expression of the triticale *ExpB4F5R3-2* mRNA occurred in mature anthers and pollen (Fig. 4). Therefore, the triticale expansin B4 mRNA appears to be regulated differently than expansin B1 which shows no expression in mature pollen (see above). However, like expansin B1, a minor larger hybridizing fragment was observed in early anther development, flowers without anthers, coleoptiles, seeds, and pistils, and may also correspond to related

expansin transcripts with a broader expression pattern (Lin et al. 2005).

The deduced proteins from the three clones *ExpB4F5R3-2*, *ExpB4F5-1*, and *ExpB4F5-2* were 98% identical. The triticale *ExpB4F5R3-2* protein showed very high sequence identity with expansin B4 from *T. aestivum*, as well as lower sequence identity to *H. lanatus*, and *D. glomerata* expansins, and group 1 grass pollen allergens from several plant species (Table S5). *ExpB4F5R3-2* displayed the same conserved domains as the expansin B1 proteins described above (Table 2). Expansin B4 belongs to the β -expansins whose role in pollen development was discussed above in the expansin B1 section.

Group 2 pollen allergens

Spots 17 and 18: Four and two different peptides were identified by MS analysis for spots 17 and 18, respectively (Table 1), and the best match was with the partial sequence of pollen allergen Lol p 2-A from *L. perenne* (30% coverage). Four triticale cDNA clones containing a corresponding complete coding sequence called *Polag2R4L-1* (Acc. No. JN388240), *Polag2R4L-2* (Acc. No. JN388241), *Polag2R4H-1* (Acc. No. JN388242), and *Polag2R4H-2* (Acc. No. JN388243) showed greater than 95% identity to each other and *Polag2R4H-2* was analyzed further. This cDNA showed homology with group 2 and group 3 grass pollen allergens with the most similarity being to barley and wheat sequences (Table S5). The highest expression of the triticale group 2 pollen allergen-related mRNA occurred in mature anthers with some transcript present in mature pollen. No expression could be detected in young anthers or in other tissues (Fig. 4). The deduced proteins *Polag2R4L-1*, *Polag2R4L-2*, *Polag2R4H-1*, and *Polag2R4H-2* displayed greater were 97% identical. *Polag2R4H-2* exhibited substantial identity to *H. vulgare* and to *L. perenne* and *D. glomerata* protein sequences (Table S5).

The group 1 pollen allergen superfamily/ α -expansin conserved domain is found in the deduced *Polag2R4H-2* protein (Table 2). The pollen allergen 1 superfamily comprises the pollen allergen groups 1, 2, and 3 (Ansari et al. 1989). It has been proposed that the C-terminus of group 1 allergens was duplicated and underwent point mutations creating group 2 and 3 allergens (Petersen et al. 2006). *Polag2R4H-2* might also be allergenic and sequence similarity with α -expansin indicates that it may be involved in regulating cell expansion by loosening the cell wall. In a simplified model, Sampedro and Cosgrove (2005) have suggested that both the α - and β -expansins cause the loosening of the cell wall, α -expansins by dissociating xyloglucans and β -expansins by dissociating xylans in cellulose microfibrils.

Group 3 pollen allergens

Spots 14 and 15: A total of seven different peptides were identified by MS analysis for spots 14 and 15 (3 and 4 peptides, respectively, Table 1), and the best match was with the putative group 3 grass pollen allergen Tri a 3 from *T. aestivum* (47% coverage). The peptides from both spots could represent the same protein which has undergone post-translational modifications. Two triticale cDNA clones containing a corresponding complete coding sequence were obtained and called *TrisA3-1* (Acc. No. JN388244) and *TrisA3-3* (Acc. No. JN388245). These clones showed 93% sequence identity to each other. *TrisA3-1* displayed high similarity to a group 3 allergen from *T. aestivum* and less so to group 2 allergens from *D. glomerata* and *C. dactylon* (Table S5). Northern blot analysis indicated that the highest level of *TrisA3-1* expression occurred in mature anthers and pollen, whereas no expression could be detected in any of the other plant tissues tested (Fig. 4).

The deduced proteins of the *TrisA3-1* and *TrisA3-3* clones were 93% identical. High sequence identity was present between the deduced *TrisA3-1* protein and Tri a 3 from *T. aestivum*, whereas significant sequence identity was found with group 2 and 3 grass pollen allergens (Table S5).

Based on sequence homology, the *TrisA3-1* belongs to group 3 pollen allergens found in a number of grasses including wheat, maize, timothy grass, and Bermuda grass (Ansari et al. 1989; Guérin-Marchand et al. 1996; Petersen et al. 2006). Sequences vary widely among members of group 3 pollen allergens and show homology with group 1 and 2 pollen allergens. Since group 3 allergens also show considerable homology with pollen expansins, it can be speculated that they might also be involved in the expansin-like activities during pollen germination and tube growth discussed above.

Group 4 pollen allergen

Spot 1: Six different peptides were identified by MS analysis (Table 1). These peptides showed 100% sequence identity with the partial sequence of pollen allergen Tri a 4 from *T. aestivum* (26% coverage). Group 4 allergens were first characterized in rye grass (*Lolium perenne*) by Ekramoddoullah et al. (1983) and are 50–67 kDa basic glycoproteins which, along with group 7, 11, and 12 allergens, are not specific to grasses (Hrabina et al. 2008). A triticale cDNA clone, *TriA4F3R4-1* (Acc. No. JN388239) was obtained and showed high level sequence identity to group 4 allergens from *T. aestivum* (100%) and other Triticeae species (Table S5). As shown in Fig. 4, a hybridization signal obtained with the *TriA4F3R4-1* probe was first

detected in 4.5 mm anthers, its expression peaked in 5.5 mm anthers and could also be observed in mature pollen. No expression could be detected in other tissues.

As expected, the *TriA4F3R4-1* derived amino acid sequence showed high sequence homology with group 4 allergens from *T. aestivum*, *S. cereale*, *H. vulgare* and *P. pratense* (Table S5). The perfect match of *TriA4F3R4-1* to the wheat Tri a 4 allele A indicates that it likely originated from the *Triticum* genomes of triticale. A study using antiserum against Phl p 4 showed that group 4-related allergens can occur in pollen of dissimilar plants like mugwort (*Artemisia vulgaris*) and birch (*Betula verrucosa*) (Grote et al. 2002). To our knowledge, this is the first report of a full-length sequence of a group 4 allergen from the Triticeae.

An FAD/FMN-containing dehydrogenase domain, a FAD-binding domain, and a berberine (BBE)-like domain were present in the *TriA4F3R4-1* protein (Table 2). BBE is an oxido-reductase involved in the biosynthesis of alkaloids in plants that catalyzes the transformation of (S)-reticuline into (S)-scoulerine (Nandy et al. 2005; Winkler et al. 2006). These alkaloids have been described to play important roles in plant defence. However, according to Nandy et al. (2005), there is no BBE enzymatic activity in Phl p 4 and therefore a role of group 4 pollen allergens in plant defence by producing antimicrobial alkaloids is still questionable. Interestingly, Grote et al. (2002) have shown that Phl p 4 was located in the pollen cell wall and in cytoplasmic P-particles (polysaccharide-containing wall-precursor bodies). Therefore group 4 allergens are well positioned to be rapidly released upon pollen hydration and may have a role in early triticale pollen-stigma interactions.

Group 5 pollen allergens

Spots 4, 5, and 22: A total of 31 peptides were identified by MS analysis for spots 4, 5, and 22 (Table 1). The peptides for spots 4 and 5 showed 100% sequence identity with group 9 pollen allergens from *T. aestivum* and *H. vulgare* (60 and 20% coverage, respectively). Peptides for spot 22 showed high sequence identity with a group 5 allergen from *T. aestivum* (44% coverage). Group 9 allergens share immunological and structural similarity with group 5 pollen allergens and were re-classified to group 5 (Olsen et al. 1991; <http://www.allergen.org>). The cDNA clone *Tris5R8-5* (Acc. No. JN578726) had considerable sequence identity to group 9 pollen allergens from *H. vulgare* and *T. aestivum* and with group 5 pollen allergens from *S. cereale* and *P. pratense* (Table S5). Northern blot analysis revealed that the triticale group 5 pollen allergen transcript was first detected in 4.5 mm anthers and peaked in mature pollen, whereas no expression could be detected in other tissues (Fig. 4). Group 5 grass pollen allergen genes have been

previously reported to express at pollen maturity (Astwood and Hill 1996).

The Tris5R8-5 derived amino acid sequence showed sequence identity with pollen allergens from a wide range of plant species (Table S5). The Tris5R8-5 deduced protein contains no putative signal peptide or N-glycosylation sites (Table 2). However, Hor v 9 from *H. vulgare* has 79.4% sequence identity with Tris5R8-5 and it has a putative 27-amino acid signal peptide, a potential N-glycosylation site, and a possible transmembrane sequence (Astwood and Hill 1996). This suggests that the Tris5R8-5 cDNA may be a partial sequence. The signal sequence of Hor v 9 and other pollen allergens have been held responsible for translocation of these proteins to the amyloplast for post-translational modifications (Singh et al. 1991; Astwood and Hill 1996). The domains identified in Tris5R8-5 include the characteristic group 5 pollen allergen ribonuclease and pollen allergen 6 domains. Whether group 5 allergens possess RNase activity remains a question (Bufe et al. 1995; Andersson and Lidholm 2003). Localization studies by Astwood and Hill (1996) have indicated that upon hydration Hor v 9 is secreted to the extracellular matrix.

Group 7 pollen allergens

Spot 20: A single peptide was identified by MS analysis (Table 1) and the best match was found to be with the full-length sequence of the calcium-binding pollen allergen Phl p 7 from *P. pratense* (15.4% coverage). Four triticales cDNA clones containing a corresponding complete coding sequence were obtained and called *G7F2-1* (Acc. No. JN388246), *G7F2-2* (Acc. No. JN388247), *G7F2-3* (Acc. No. JN388248), and *G7F2-4* (Acc. No. JN388249). These clones showed greater than 94% identity to each other and *G7F2-3* showed sequence identity with group 7 allergens from *O. sativa*, *Sorghum bicolor*, and *P. pratense* (Table S5). Upon northern blot analysis, the *G7F2-3* probe hybridized with two bands of similar size found principally in mature anthers. No expression could be detected in the anthers at earlier stages of development or in other tissues (Fig. 4).

The deduced proteins from all four cDNA clones showed greater than 97% identity to each other. The *G7F2-3* sequence encodes a 78 amino acid protein with a predicted molecular weight of 8.7 kDa and pI 4.2. These values are similar to those observed on the 2-D gel for spot 21 supporting the possibility that it corresponds to the group 7 allergen in spite of the limited MS data. The triticales *G7F2-3* protein displayed very high sequence identity with the calcium-binding group 7 pollen allergens from *P. pratense*, *O. sativa*, and *C. dactylon* (Table S5). Two calcium-binding α -helices called EF-hand domains are present in the *G7F2-3* deduced protein (Table 2). Several

pollen allergens from various plants have been identified as calcium-binding proteins containing EF-hand domains (Valenta et al. 1998). These proteins are called polcalcins because of their pollen-specific expression and their calcium-binding property (Ledesma et al. 2006). Previously described polcalcins include a two EF-hand Phl p 7 from *P. pratense* (Niederberger et al. 1999), a two EF-hand Bet v 4 from *B. verrucosa* (Grote et al. 1999), a four EF-hand Ole e 8 from *O. europaea* (Ledesma et al. 2000), and the two EF-hand Bra r 1 and Bra r 2 from *Brassica rapa* (Okada et al. 1999). An EF-hand calcium-binding protein has also been reported in the tryphine of *Arabidopsis thaliana* pollen by Mayfield et al. (2001).

These proteins are highly conserved which may reflect their important role in pollen development, germination, and/or tube growth. It is speculated that these proteins may serve as calcium reservoirs. Since the conformation of polcalcins changes by binding calcium, these proteins are also thought to be involved in intracellular signalling (Grote et al. 2008). In a study based on the immunolocalization of polcalcins Bra r 1 and Bra r 2 from *B. rapa*, Okada et al. (1999) found that these proteins accumulate in mature pollen and were easily released upon pollen hydration. In addition, these proteins were present in elongating pollen tubes and they hypothesized that they might play a role in pollen-pistil interactions and pollen tube growth.

Group 11 pollen allergens

Spots 11 and 12: Four different peptides were identified by MS analysis for spot 11 (Table 1) and the best match was to the pollen allergen Phl p11 from *P. pratense* (13% coverage). For spot 12, five different peptides were identified matching Lol p 11 from *L. perenne* (20% coverage). Five cDNA clones from triticales containing a corresponding complete coding sequence were obtained and called *Tris11F7-1* (Acc. No. JN578703), *Tris11F7-4* (Acc. No. JN578704), *Tris11F7-6* (Acc. No. JN578705), *Tris11F8-6* (Acc. No. JN578706), and *Tris11F8-9* (Acc. No. JN578707). Another cDNA clone named *Tris11R1-2* (Acc. No. JN578708) containing a partial coding sequence was also obtained. These cDNA clones showed greater than 99.4% sequence identity and the *Tris11F8-9* clone was analyzed further. This cDNA displayed significant identity with group 11 pollen allergens from *P. pratense* and *O. sativa*, and an mRNA sequence from *H. vulgare* (Table S5). Northern blot analysis indicated that the transcript hybridizing to *Tris11F8-9* was first detected in 4.5 mm anthers with high expression in mature anthers. A transcript was observed in mature pollen, but not in any of the other plant tissues tested (Fig. 4).

The deduced protein sequences Tris11F7-1, Tris11F7-4, Tris11F7-6, Tris11F8-6, and Tris11F8-9 have a minimum of 95% sequence identity with each other. A significant level of sequence identity was found between Tris11F8-9 and group 11 pollen allergens (Table S5).

Group 11 grass pollen allergens show sequence homology and structural similarity to the soybean trypsin-inhibitor-like family, however, Lol p 11 from *L. perenne* showed no inhibitory activity towards trypsin (Van Ree et al. 1995). A pollen Ole e 1 allergen/extensin domain was found in Tris11F8-9 protein (Table 2). Group 11 pollen allergens such as Phl p 11, Lol p 11, and the pollen-specific protein C13 precursor from *Z. mays* also have sequence homology with the olive pollen allergen Ole e 1/extensin family (Andersson and Lidholm 2003; Van Ree et al. 1995). Extensins are important constituents of primary cell walls and maintain cell wall integrity (Gupta et al. 1996). Van Ree et al. (1995) have indicated that group 11 pollen allergens are glycoproteins and a considerable part of the IgE reactivity of Lol p 11 can be attributed to the glycan moiety. Antibodies directed against this glycan moiety cross-reacted with horseradish peroxidase, bromelain, and ascorbate oxidase (Weber 2007).

Based on the results from in vitro germination of olive pollen grains, Alché et al. (2004) have proposed that Ole e 1, through signal transduction, is involved in controlling the pre-germination of pollen and the emergence and guidance of pollen tube. Pollen-specific proteins including Zmc13 from maize and LAT52 from tomato contain Ole e 1 domains and are described to play an important role in pollen hydration as well as in pollen germination (Hanson et al. 1989; Jiang et al. 2005; Dai et al. 2006). Since Tris11F8-9 contains an Ole e I domain it may have a similar function. The triticales Tris11F8-9 represents the first example of a Triticeae Phl p 11-like protein.

Pollen-specific allergen

Spot 13: Four different peptides were identified by MS analysis (Table 1) and the best match was to a pollen-specific gene from *O. sativa* (23.2% coverage). Three triticales cDNA clones, *PolspecF8-4* (Acc. No. JN578709), *PolspecF8-5* (Acc. No. JN578710), and *PolspecF8-6* (Acc. No. JN578711) containing a corresponding complete coding sequence were obtained. These clones showed greater than 99% identity to each other and the *PolspecF8-5* clone matched sequences from *T. aestivum* and *H. vulgare* and the pollen-specific protein C13 from *O. sativa* (Table S5). Northern blot analysis indicated that expression of the triticales pollen-specific allergen mRNA was abundant in mature anthers and pollen (Fig. 4). Transcript of *PolspecF8-5* was first found in 4.5 mm anthers and no expression could be detected in any of the other plant tissues tested.

The deduced proteins from the three triticales cDNA clones were 99.8% identical and matched perfectly all 4 peptides initially identified by MS analysis. Although *PolspecF8-5* showed limited identity (34%) to Group 11 pollen allergens discussed above, it showed high sequence identity with pollen Ole e 1-related proteins (Table S5). Accordingly, a conserved pollen Ole e 1/extensin domain is present in the *PolspecF8-5* deduced protein (Table 2). Pollen Ole e 1/extensin domain containing proteins and their role in pollen germination has been discussed in the previous section on group 11 pollen allergens.

Polygalacturonase

Spot 2: One peptide was identified by MS analysis (Table 1) and it showed perfect identity with the *Zea m 13* allergen from *Z. mays* (2.7% coverage) which is a polygalacturonase. Polygalacturonases from the majority of common grasses have been classified as group 13 allergens sharing high sequence homology and IgE cross-reactivity (Swoboda et al. 2004). The catalytic site of these enzymes is highly conserved (Petersen et al. 2006). A triticales cDNA clone containing a perfect match to the identified peptide was obtained and called *PGR2-7* (Acc. No. JN578712). It shared considerable identity with other grass polygalacturonases and with the group 13 allergen from *Paspalum notatum* and *Z. mays* (Table S5). Northern blot analysis indicated that expression of the putative triticales polygalacturonase began in 4.5 mm and peaked in 5.5 mm anthers and mature pollen (Fig. 4). No significant expression could be detected in young anthers or in the other plant tissues. Similarly, lily and barley pollen-specific polygalacturonase genes were shown to express in developing pollen and accumulate in mature pollen (Chiang et al. 2006; Pulido et al. 2009).

The best matches to the derived *PGR2-7* amino acid sequence were to proteins from *H. vulgare* and *P. pratense* (Table S5). *PGR2-7* corresponds to a protein of 415 amino acids with a signal peptide of 22 amino acid residues. The deduced molecular weight and pI of the mature *PGR2-7* protein are 41.45 kDa and 7.01, whereas the observed molecular weight and pI were approximately 72 kDa and 7, respectively (Table 2). This discrepancy might be due to glycosylation (Table 2), although it is possible that spot 2 does not represent the polygalacturonase protein even though the cDNA clone corresponds to a polygalacturonase gene expressed in pollen.

A glycosyl hydrolase family-28 domain found in polygalacturonases could be identified in the deduced *PGR2-7* protein (Table 2). Polygalacturonases are members of a large gene family with diverse functions (Kim et al. 2006). Polygalacturonases are thought to provide wall precursors for pollen tube growth. These enzymes are also

considered to facilitate the penetration of the pollen tube into the stigma and style tissues by degrading their cell walls (Chiang et al. 2006; Niogret et al. 1991). These possibilities are consistent with the fact that the Phl p 13 polygalacturonase allergen from *P. pratense* has been detected in the pollen cell wall and P-particles (Grote et al. 2005) and the *Brassica napus* pollen polygalacturonase was shown to occur in the cell wall and pollen tube (Dearnaley and Daggard 2001). The identification of this enzyme within rapidly released triticale pollen proteins is consistent with these observations. Identification of this important pollen enzyme for the first time in a member of the Triticeae should help further studies characterizing its role.

Glucan endo- β -1,3-glucanase

Spot 6: One peptide was identified by MS (Table 1) and the best match was to a β -1,3-glucanase from *T. aestivum* (4.8% coverage). Six triticale cDNA clones containing complete coding sequences were obtained and called *GlucanR3-1* (Acc. No. JN578713), *GlucanR3-2* (Acc. No. JN578714), *GlucanR3-3* (Acc. No. JN578715), *GlucanR3-4* (Acc. No. JF811350), *GlucanR3-5* (Acc. No. JN578716), and *GlucanR3-6* (Acc. No. JN578717). These cDNA clones showed greater than 99.4% identity to each other and cDNA clone *GlucanR3-4* displayed high identity with other Triticeae endo- β -1,3-glucanases (Table S5). The putative triticale glucan endo- β -1,3-glucanase *GlucanR3-4* transcript was first detected in 4.5 mm anthers and increased in 5.5 mm anthers and mature pollen grains, but did not occur in other tissues (Fig. 4). A weaker signal of smaller size was also observed in 1.5 and 2.5 mm anthers, as well as the stigma.

The deduced proteins from the six triticale cDNA clones were more than 98% identical and all six sequences encode a 336 amino acid protein. The *GlucanR3-4* deduced protein exhibits a high degree of sequence identity to Triticeae glucan endo- β -1,3-glucanases (Table S5). Perfect identity to the wheat sequence would suggest that the *GlucanR3-4* gene originates from the *Triticum* genomes of triticale. The *Glucan R3-4* protein has a signal sequence of 31 amino acid residues. The predicted molecular weight and pI of the mature protein are 32.15 kDa and 4.79, respectively, whereas the observed molecular weight and pI on the 2D gel were 32 kDa and 4.8, respectively. This indicates that spot 6 may indeed correspond to a β -1,3-glucanase despite the fact that only a single MS peptide was obtained. The glycosyl hydrolase family-17 domain found in glucanases is present in the *GlucanR3-4* protein (Table 2). The β -1,3-glucanases are widely distributed in higher plants (Huecas et al. 2001; Wagner et al. 2007) and exist as structural isoforms of various sizes, isoelectric points, cellular

localization, and expression pattern (Petrucelli et al. 1999). They are reported to have a role in carbohydrate metabolism and may be involved in cell wall modification through hydrolysis of glycosidic bonds (Dai et al. 2006). During pollen germination, β -1,3-glucanases enhance pollen tube elongation by influencing the plasticity of the pollen tube tip wall (Roggen and Stanley 1969; Wagner et al. 2007). They are also believed to hydrolyze the stigma wall facilitating the penetration of pollen tube into the stigma and style tissues (Suen et al. 2003).

The weak hybridizing bands of smaller size observed in this study upon northern blot analysis could represent different but related endo- β -1,3-glucanase genes, including those known to be expressed in the tapetum and stigma. During the early stage of pollen development several exo- and endo-1,3- β -glucanases expressed in the tapetum hydrolyze the callose wall of the microspore tetrad (Suen et al. 2003). The activity of stigma-produced β -1,3-glucanases has been investigated by Wakelin and Leung (2009) in petunia. They, in conjunction with Verhoeven et al. (2005), hypothesized that β -1,3-glucanases produced in stigma tissues help pollen germinate.

A pollen allergen Ole e 9 from olive has been reported to be an endo- β -1,3-glucanase (Huecas et al. 2001). Limited similarity was observed between the olive Ole e 9 (gi: 14279168) and the triticale β -1,3-glucanase *GlucanR3-4*, with the highest identity (39%) occurring within the conserved glycosyl hydrolase-17 domain. It is difficult to speculate if the triticale β -1,3-glucanase is allergenic at this point.

Actin depolymerizing factor

Spot 10: Four different peptides were identified by MS analysis (Table 1) and the best match was to a putative actin depolymerizing factor (ADF) from *O. sativa* (29% coverage). Three triticale cDNA clones containing a corresponding complete coding sequence were obtained and called *ADF4-1* (Acc. No. JN578718), *ADF4-2* (Acc. No. JN578719), and *ADF4-5* (Acc. No. JF811346). These cDNA clones showed greater than 94% identity to each other. *ADF4-5* displayed strong identity to genes from *S. bicolor* and *H. vulgare*, and to an *ADF* gene from *Z. mays* (Table S5). Northern blot analysis showed that the expression of the putative triticale *ADF* gene peaked in mature anthers and mature pollen, whereas no expression could be detected in the other plant tissues tested (Fig. 4).

The deduced proteins from triticale cDNA clones *ADF4-1*, *ADF4-2*, and *ADF4-5* were 97% identical and displayed a high degree of sequence identity to other cereal actin depolymerizing factor/cofilin-like proteins (Table S5). In plants, ADFs have been grouped in two different classes: vegetative and reproductive, with reproductive

ADFs reported in Arabidopsis, maize, lily, Brassica, tobacco, and rice (Feng et al. 2006). The triticale ADF isolated in the present study is therefore the first characterized reproductive ADF from a member of the Triticeae.

The ADF4-5 has a putative actin-binding domain (Table 2) and a putative serine phosphorylation site. ADF is a low-molecular-weight protein which binds to F- and G-actin and is involved in the organization of the cytoskeleton by regulating actin dynamics (Li et al. 2010; Staiger et al. 2010; Tian et al. 2009). In maturing pollen, ADF binds to the actin assembly breaking it down into an actin-ADF complex which serves as the storage form of actin in dehydrated pollen (Hussey et al. 2002). In germinating pollen, the actin-ADF complex breaks down into ADF and actin, and a new actin assembly is formed (Hussey et al. 2002; Smertenko et al. 2001). ADF activity during pollen tube growth is modulated by the phosphorylation of Ser6 present at the N-terminus (Smertenko et al. 1998). Pollen ADF from lily, however, is not regulated by phosphorylation (Allwood et al. 2002). It will be interesting to find out if the triticale ADF4-5 requires phosphorylation for its regulation.

Pollen profilin

Spot 19: Three different peptides were identified by MS analysis (Table 1) and the best match was with profilin 3 from *P. pratense* (26% coverage). Plant profilins belong to a divergent multigene family of low-molecular weight proteins and occur in different kinds of cells (Kovar et al. 2000). Two different types of profilins have been identified in maize and Arabidopsis, one type is expressed in vegetative and reproductive tissues at various developmental stages, while the other type is expressed in pollen late in development (Kovar et al. 2000; Ren and Xiang 2007). In the present study, we also identified the more broadly expressed triticale profilin which will be discussed below. A cDNA clone encoding a complete coding sequence was obtained from triticale and called *Prof3R4-2* (Acc. No. JN578720). It displayed high sequence identity with profilin from *P. pratense* (Table S5). Northern blot analysis indicated that the triticale pollen *Prof3R4-2* profilin transcript displayed an expression pattern similar to several other triticale genes described above with expression becoming evident in 4.5 mm anthers and peaking in mature anthers and pollen, whereas no signal could be detected in tissues other than anthers (Fig. 4).

High sequence identity was present between the *Prof3R4-2* deduced protein and profilin sequences from a broad variety of species (Table S5). A profilin domain, poly-proline binding sites, actin interaction sites, and putative PIP2-interaction sites are present in the *Prof3R4-2* protein (Table 2). At high concentrations, profilin inhibits the rate of polymerization of actin, whereas at low

concentrations profilin enhances this polymerization (Yarmola et al. 2008). Profilins are speculated to regulate the dynamics of the pollen actin cytoskeleton in germinating pollen (Kandasamy et al. 2007). The activity of profilin is regulated by Ca^{2+} in the cytosol (Ren and Xiang 2007). It has been shown that in the growing pollen tubes of *Lilium longiflorum*, profilin is evenly distributed throughout the cytoplasm (Vidali and Hepler 1997). It is unclear why profilin was detected upon pollen hydration. It can be speculated that profilins could be playing a role in calcium binding at the pollen-stigma interface, in addition to their role in pollen tube growth. The triticale *Prof3R4-2* is the first Triticeae pollen-specific profilin described and it will be interesting to investigate whether it interacts with the above ADF to regulate actin dynamics.

Pollen profilins are members of a conserved family present in all eukaryotic cells and are classified as group 12 allergens (de Weerd et al. 2002; Hrabina et al. 2008). The highly conserved conformational structure of profilins has been described to be responsible for immunological cross-reactivity among different profilins from fruits, pollen, and humans (Sankian et al. 2005).

Profilin

Spot 21: Two different peptides were identified by MS analysis (Table 1) and the best match was with a profilin sequence from *H. vulgare* (17.6% coverage). The corresponding triticale cDNA clone containing a complete coding sequence was called *ProfR2-2* (Acc. No. JN578721). It showed a high level of identity with other cereal profilin sequences (Table S5). Northern blot analysis showed this triticale profilin transcript to be present in all tissues tested (Fig. 4).

A high degree of sequence identity was present between the *ProfR2-2* deduced protein and profilin sequences from *H. vulgare*, *T. aestivum*, and *P. pratense* (Table S5). The deduced *ProfR2-2* protein possessed the same domains (Table 2) as the pollen-specific *Prof3R4-2* profilin described above and shared 79.4% amino acid identity with it. In Arabidopsis, despite the fact that the pollen and constitutively expressed profilins share 77% amino acid sequence identity and have the same domains, they are structurally and functionally different from each other (Kandasamy et al. 2007). The constitutively expressed profilins bind to G-actin and poly-proline with higher affinity and are more sensitive to the presence of Ca^{2+} than the pollen profilins (Gibbon et al. 1998; Kovar et al. 2000; Ren and Xiang 2007).

Cyclophilin

Spot 8: Eight different peptides were identified by MS analysis (Table 1) and the best match was with cyclophilin

A-2 protein from *T. aestivum* (53% coverage). The triticales cDNA clone *CycloF4R-1* (Acc. No. JF811347) displayed 100% identity with a *T. aestivum* cyclophilin A (Table S5) and the corresponding transcript was detected in all plant tissues tested (Fig. 4) including mature pollen (upon longer exposure, results not shown).

The perfect match between the deduced CycloF4R-1 protein and a wheat cyclophilin (Table S5) suggests that the triticales sequence likely originated from the *Triticum* genomes. A putative cyclophilin-type peptidyl-prolyl cis-trans isomerase domain is present in CycloF4R-1 protein (Table 2).

In plants, cyclophilins, also known as peptidyl-prolyl cis-trans isomerases, belong to a group of stress-induced proteins responding to various environmental stimuli including wounding and exposure to chemicals. It has been suggested that they can serve as molecular chaperones during various environmental stresses (Romano et al. 2004). Given that cyclophilin is relatively abundant in the triticales hydrated pollen extract (Fig. 3), in spite of the low abundance of its transcript (Fig. 4), it may serve a protective role during pollen dehydration. Cyclophilins exist in multiple isoforms and are expressed in vegetative and floral parts including roots, leaves, stems, buds, anthers, and in endosperm (Cadot et al. 2000; Johnson et al. 2001). Based on the observations that cyclophilin-type peptidyl-prolyl cis-trans isomerases are important signal molecules in animals, cyclophilins released from rice pollen have been speculated to be involved in signal transduction during pollen tube growth in the style (Dai et al. 2006).

A 21 kDa cyclophilin with peptidyl-prolyl cis-trans isomerase activity released from lily and tobacco pollen grains has been found to be immunologically reactive (Yokota et al. 2004). Similarly, cyclophilins from pollen of ryegrass, carrots, and birch have been reported to be IgE reactive (Cadot et al. 2000; Fujita et al. 2001; De Canio et al. 2009). The two allergenic peptides “DFTAGNGTGGESIYGAK” and “DXXXXXXXXTGPGILSMANAGPGTNG” from birch (Cadot et al. 2000) have 88 and 89% sequence identity, respectively, with CycloF4R-1 suggesting that it could also be allergenic.

Glutathione peroxidase

Spot 9: Four different peptides were identified by MS analysis (Table 1) and the best match was to a putative cytosolic glutathione peroxidase from *T. monococcum* (36% coverage). Two triticales cDNA clones containing a corresponding complete coding sequence and showing 95% sequence identity with each other were obtained and called *Glutpx1* (Acc. No. JN578722) and *Glutpx2* (Acc. No. JN578723). *Glutpx2* shared high identity with the glutathione peroxidase-like sequences from various species

(Table S5). Northern blot analysis indicated that the triticales *Glutpx2* transcript was present in all plant tissues tested with the highest expression levels observed in roots, stigmas, and 2.5–3.5 mm anthers (Fig. 4).

The deduced protein sequences of *Glutpx1* and *Glutpx2* are 98.8% identical and the perfect match of *Glutpx2* with a *T. monococcum* sequence (Table S5) suggests that it likely came from the *Triticum* genomes of triticales. The *Glutpx2* protein has a putative glutathione peroxidase domain (Table 2).

Glutathione peroxidases protect plants from damage by increasing tolerance to oxidative stress (Halušková et al. 2009). They remove reactive oxygen species, as well as lipid and alkyl peroxides (Noctor et al. 2002). There is still very little known of the role of glutathione peroxidases in pollen although they have been proposed to regulate pollen tube growth and wall formation, and to play a role in the interaction between pollen and pistil (Bredemeijer 1984). Several putative glutathione peroxidases have been identified in the mature pollen of *Arabidopsis*, however, like *Glutpx2*, their expression is not pollen-specific (Holmes-Davis et al. 2005). Glutathione peroxidases have also been shown to be expressed in germinating *Arabidopsis* pollen and have been classified as defence/stress response genes (Zou et al. 2009). The triticales glutathione peroxidase identified in the present study might have a role in protecting the pollen against stresses. The reason for its rapid release upon hydration, especially given the lack of a putative signal peptide, is currently unknown.

Glutaredoxin

Spot 16: Four different peptides were identified by MS analysis (Table 1) and the best match was to a glutaredoxin (GRX) from *T. aestivum* (39% coverage). Three cDNA clones from triticales containing a corresponding complete coding sequence were obtained and called *Glut4H1* (Acc. No. JN578724), *Glut4H2* (Acc. No. JF811351), and *Glut4H6* (Acc. No. JN578725). These clones showed greater than 98% identity to each other. *Glut4H2* showed sequence identity with glutaredoxin genes from *T. aestivum*, *Z. mays*, *H. vulgare*, and *Deschampsia antarctica* (Table S5). Northern blot analysis revealed relatively low expression of the transcript in all the plant tissues tested (Fig. 4), including mature anthers, even though it represented a major pollen protein spot (Fig. 3).

The deduced protein sequences from *Glut4H1*, *Glut4H2*, and *Glut4H6* have a minimum of 98.5% sequence identity with each other and *Glut4H2* showed high identity to other cereal glutaredoxins (Table S5). A putative glutaredoxin domain, putative glutathione (GSH) binding sites, and a thioltransferase activity site are present in the *Glut4H2* deduced protein (Table 2).

Glutaredoxins reduce intracellular disulfide bonds using conserved cysteines located in the active site motif and depend on glutathione (reviewed by Meyer et al. 2009). The *A. thaliana* GRX family comprises 31 members which fall into three subgroups each characterized by a particular thioltransferase active site motif: CPYC, CGFS, or CC-type (Rouhier et al. 2004). There are two major catalytic mechanisms used by glutaredoxins; a dithiol and a monothiol reaction. GRXs with the active site CXXC, like the putative triticale Glut4H2 GRX, are able to catalyze both reactions. The role of GRXs is critical in DNA synthesis, signal transduction, and the defence against oxidative stress by regulating the redox state of proteins (Kang et al. 2007). The function of GRXs is critical in pollen development (Xing and Zachgo 2008). In transgenic Arabidopsis, CC-type GRXs (ROXY1 and ROXY2) have been shown to affect the activation of SPOROXYTELESS/NOZZLE and DYSFUNCTIONAL TAPETUM1 genes that are involved in microsporogenesis and tapetum development, respectively (Xing and Zachgo 2008; Irish 2010). In monocots such as rice, a ROXY1 homolog has been reported to have the same function in floral organ development as in Arabidopsis (Wang et al. 2009). It is unknown why a glutaredoxin would be rapidly released upon pollen hydration and it will be interesting to find out the role of triticale GRX in floral processes.

In summary, we have developed a method that greatly enriched for rapidly released triticale pollen proteins upon hydration. A short exposure to an aqueous buffer resulted in a rapid release of numerous pollen proteins for which a 2D electrophoretic profile was generated and the major proteins characterized by MS. These proteins are likely to be at the forefront of pollen/stigma interactions and, given that many correspond and are still only classified as pollen allergens, they evidently are also prominent in interactions with the immune system. This study is unique in that the major proteins released upon pollen hydration were not only identified by mass spectrometry, but the sequence and expression data for the corresponding genes were described concomitantly. In addition, proteins such as group 11 pollen allergens, polygalacturonase, and actin depolymerizing factor were characterized for the first time in the Triticeae.

Pollen cell wall proteins may originate from the tapetum or from within the developing pollen grain. Although it is difficult to ascertain the contribution of the tapetum in the absence of in situ mRNA hybridization or subcellular immunocytochemistry, many of the triticale proteins characterized in this study were found to express specifically during late pollen development at a stage at which the tapetum has degenerated. However, there were a number of abundant proteins for which very little mRNA was detected in mature pollen suggesting that either sufficient protein is

stored during pollen development or that the protein is expressed in the tapetum and subsequently transferred to the pollen grain. In either case, there is apparently no need for their rapid translation during the early events associated with pollen germination. On the other hand, for some of the proteins characterized, there appears to be a need for both high amounts of mRNA and protein within the mature pollen grain.

Most of the proteins displayed anther/pollen-specific expression patterns consistent with specialized functions in pollen development and germination. Many of the proteins identified possessed features in line with a rapid release from the pollen grain and these included the presence of a signal peptide, a demonstrated subcellular localization to the cell wall, or putative functions deemed consistent with pollen germination and tube growth, such as cell wall modification. However, not all proteins displayed such features and although it is possible that some of these might still represent contamination by abundant cytoplasmic proteins, further studies will be required to establish whether they truly serve a specific role in pollen-stigma interactions. Lastly, some triticale genes were identical to *Triticum* genes whereas no such perfect matches were found to *Secale* sequences, it will be interesting to establish whether this is simply a reflection of the larger number of publicly available *Triticum* sequences or whether it points to genetic or epigenetic modifications within the triticale allopolyploid which possesses a self-pollination system closer to that of wheat.

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Table S1. Primer sequences for triticale gene PCR amplification.

Gene	Primers		PCR Product Length (bp)
<i>Expansin B1</i>	ExpB5-F4	CACTCACAAAGTCACAACAAACACGA	1104
	3'RACE outer	GCGAGCACAGAATTAATACGACT	
<i>Expansin B4</i>	ExpB4-F5	GACAGCGGCAACAAGGAACAGTTCAAG	1076
	ExpB4-R3	GCGTGTATACCAATTTCTATTAATAAAAC	
<i>Group 2 pollen allergen</i>	Pol allerg2-R4	AATCGAGACAACACGTGCACTGGA	701
	5'RACE inner	CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG	
<i>Group 3 pollen allergen</i>	TriA3-R1	CAAACATGTCCGTCGTTGCTTCTC	629
	5'RACE outer	GCTGATGGCGATGAATGAACACTG	
<i>Group 4 pollen allergen</i>	TriA4-F3	AAACCACACGGTCACACAACACAC	1750
	TriA4-R4	TAATTCATAAATAAAGTGGCAGATTCC	
<i>Group 5 pollen allergen</i>	Phlp5-R8	CAGGCGCATGCACACGCGGTCGATTTC	972
	5'RACE outer	GCTGATGGCGATGAATGAACACTG	
<i>Group 7 pollen allergen</i>	G7-F2	ACCGATCGAAGAGTGATTCGAGGT	703
	3'RACE outer	GCGAGCACAGAATTAATACGACT	
<i>Group 11 pollen allergen</i>	Phlp11F8	CCCCGCCCCGCCGCTTGTTG	847
	3'RACE outer	GCGAGCACAGAATTAATACGACT	
<i>Pollen-specific allergen</i>	Polspec-F8	CAGCCTCCGCCGAGCCAGAACACA	853
	3'RACE outer	GCGAGCACAGAATTAATACGACT	
<i>Polygalacturonase</i>	Polygala R2	CGGATTAGCGGTTAGATGTGAGATGAC	1573
	5'RACE outer	GCTGATGGCGATGAATGAACACTG	
<i>Endo-β-1,3-glucanase</i>	GlucanF4	TGAATGAACACTGCGTTTGCTGGC	1281
	GlucanR3	GAATCAAAAGCTGTACATCACAAGTT	
<i>Actin depolymerizing factor</i>	ADF-4	GGAAGGAAGGAAACAAGAGAAAGCATCA	797
	3'RACE outer	GCGAGCACAGAATTAATACGACT	
<i>Pollen profilin</i>	Prof3-R4	TGCATTGCAATCAAAGAGGCCAT	872
	5'RACE outer	GCTGATGGCGATGAATGAACACTG	
<i>Profilin</i>	Prof-R2	CAATAAAGCAGCAGCAGAACCCACAAGT	840
	5'RACE outer	GCTGATGGCGATGAATGAACACTG	
<i>Cyclophilin</i>	CycloF4	TCACAGAGCCCCGAAGCCAAAGGAA	919

	3'RACE inner	CGCGGATCCGAATTAATACGACTCACTATAGG	
<i>Glutathione peroxidase</i>	Glut peroxR2	GCTGGGTACAGATTTTATTTCAACAC	791
	5'RACE outer	GCTGATGGCGATGAATGAACACTG	
<i>Glutaredoxin</i>	Glut-F4	GTGCCAGACGTGCCACGC	778
	3'RACE outer	GCGAGCACAGAATTAATACGACT	

Table S2. List of cDNA clones corresponding to tritcale pollen proteins quickly released upon hydration.

cDNA clone	Accession No.	Protein	Spot No.
<i>TriA4F3R4-1*</i>	JN388239	Group 4 pollen allergen	1
<i>PGR2-7*</i>	JN578712	Polygalacturonase	2
<i>ExpB4F5-1</i>	JN578728	Expansin B4	3
<i>ExpB4F5-2</i>	JN578729	Expansin B4	3
<i>ExpB4F5R3-2*</i>	JF811349	Expansin B4	3
<i>Tris5R8-5*</i>	JN578726	Group 5 pollen allergen	4,5,22
<i>GlucanR3-1</i>	JN578713	Glucan endo- β -1,3-Glucanase	6
<i>GlucanR3-2</i>	JN578714	Glucan endo- β -1,3-Glucanase	6
<i>GlucanR3-3</i>	JN578715	Glucan endo- β -1,3-Glucanase	6
<i>GlucanR3-4*</i>	JF811350	Glucan endo- β -1,3-Glucanase	6
<i>GlucanR3-5</i>	JN578716	Glucan endo- β -1,3-Glucanase	6
<i>GlucanR3-6</i>	JN578717	Glucan endo- β -1,3-Glucanase	6
<i>ExM1</i>	JN578727	Expansin B1	7
<i>ExM2*</i>	JF811348	Expansin B1	7
<i>CycloF4R-1*</i>	JF811347	Cyclophilin	8
<i>Glutpx1</i>	JN578722	Glutathione peroxidase	9
<i>Glutpx2*</i>	JN578723	Glutathione peroxidase	9
<i>ADF4-1</i>	JN578718	Actin Depolymerizing Factor	10
<i>ADF4-2</i>	JN578719	Actin Depolymerizing Factor	10
<i>ADF4-5*</i>	JF811346	Actin Depolymerizing Factor	10
<i>Tris11F7-1</i>	JN578703	Group 11 pollen allergen	11,12
<i>Tris11F7-4</i>	JN578704	Group 11 pollen allergen	11,12
<i>Tris11F7-6</i>	JN578705	Group 11 pollen allergen	11,12
<i>Tris11F8-6</i>	JN578706	Group 11 pollen allergen	11,12
<i>Tris11F8-9*</i>	JN578707	Group 11 pollen allergen	11,12
<i>Tris11R1-2</i>	JN578708	Group 11 pollen allergen	11,12
<i>PolspecF8-4</i>	JN578709	Pollen-specific allergen	13
<i>PolspecF8-5*</i>	JN578710	Pollen-specific allergen	13
<i>PolspecF8-6</i>	JN578711	Pollen-specific allergen	13
<i>TrisA3-1*</i>	JN388244	Group 3 pollen allergen	14,15
<i>TrisA3-3</i>	JN388245	Group 3 pollen allergen	14,15
<i>Glut4H1</i>	JN578724	Glutaredoxin	16
<i>Glut4H2*</i>	JF811351	Glutaredoxin	16
<i>Glut4H6</i>	JN578725	Glutaredoxin	16
<i>Polag2R4L-1</i>	JN388240	Group 2 pollen allergen	17,18
<i>Polag2R4L-2</i>	JN388241	Group 2 pollen allergen	17,18
<i>Polag2R4H-1</i>	JN388242	Group 2 pollen allergen	17,18
<i>Polag2R4H-2*</i>	JN388243	Group 2 pollen allergen	17,18
<i>Prof3R4-2*</i>	JN578720	Pollen profilin	19
<i>G7F2-1</i>	JN388246	Group 7 pollen allergen	20
<i>G7F2-2</i>	JN388247	Group 7 pollen allergen	20
<i>G7F2-3*</i>	JN388248	Group 7 pollen allergen	20
<i>G7F2-4</i>	JN388249	Group 7 pollen allergen	20
<i>ProfR2-2*</i>	JN578721	Profilin	21

*Representative cDNA clone used in public sequence database searches

Table S3. Comparisons between MS peptide sequences and deduced amino acid sequences from cDNA clones.

cDNA clone	Protein	MS Peptides	Deduced peptides ^a
<i>Polag2R4H-2</i>	Group 2 pollen allergen	NVFDDVVP ~~I~~ PTDFK PPVSFTVEK EEWLPLKK VGTTY	NVFDDV ~~I~~ PTDFK PPVSITVEK <u>ED</u> WLPLKK <u>C</u> GTTY
<i>Tris5R8-5</i>	Group 5 pollen allergen	KAIDQVDAAFR KLSSSLELSYKL KFTVFQTALSKA KQAYAATVAAAP ~~E~~ VK KYDAYVATLTESLR KAQGATPEAKY KFIPALEAAVKQ	KAQGATPE <u>I</u> KY
<i>Tris11F8-9</i>	Group 11 pollen allergen	ECGGILQAYDL SPDASCAEIEEF ~~R~~ VEGATVAMDCR VPLTSNDGMK	VPLTSN <u>N</u> GMK

^aAmino acid differences are shown in bold and underlined.

Table S4. DNA probes used for northern blot analyses.

Gene	Spot No.	cDNA clone	Probe size (bp)	Restriction enzymes
<i>Expansin B1</i>	7	pExM2	~1085	<i>Eco</i> RI
<i>Expansin B4</i>	3	p3ExpB4-2	760	<i>Eco</i> RI
<i>Group 2 pollen allergen</i>	17,18	p3Polag2 F1-1	665	<i>Eco</i> RI
<i>Group 3 pollen allergen</i>	14,15	p5TriA3-1	653	<i>Eco</i> RI
<i>Group 4 pollen allergen</i>	1	p5TriA4 5.1	1106	<i>Eco</i> RI
<i>Group 5 pollen allergen</i>	4,5,22	p5Phlp5R2-3	785	<i>Nde</i> I + <i>Sac</i> II
<i>Group 7 pollen allergen</i>	20	p3G7F2-3	723	<i>Eco</i> RI
<i>Group 11 pollen allergen</i>	11,12	p3Phlp11F8-9	798	<i>Nde</i> I
<i>Pollen-specific protein</i>	13	p3PolspecF8-5	890	<i>Sac</i> II + <i>Nde</i> I
<i>Polygalacturonase</i>	2	p5PolygalaR2-7	~801	<i>Sma</i> I + <i>Sac</i> II
<i>Glucan endo-β-1,3-Glucanase</i>	6	p3GlucanF4-4	~1400	<i>Eco</i> RI
<i>Actin depolymerizing factor</i>	10	p3ADF4-5	783	<i>Eco</i> RI + <i>Pst</i> I
<i>Pollen profilin</i>	19	p5Prof3R4-2	288	<i>Bcl</i> I*
<i>Profilin</i>	21	p5ProfR2-1	871	<i>Eco</i> RI
<i>Cyclophilin</i>	8	p3Cyclo4	607	<i>Eco</i> RI
<i>Glutathione peroxidase</i>	9	p5Glutpx4	723	<i>Eco</i> RI
<i>Gluteradoxin</i>	16	p3Glut4H2	802	<i>Eco</i> RI

*PCR amplified product using Prof3-F4 + 5' RACE inner primer was digested with *Bcl* I to yield a 288 bp probe fragment (position 538-825, acc. No. JN578720).

Table S4. DNA probes used for northern blot analyses.

Gene	Spot No.	cDNA clone	Probe size (bp)	Restriction enzymes
<i>Expansin B1</i>	7	pExM2	~1085	<i>Eco</i> RI
<i>Expansin B4</i>	3	p3ExpB4-2	760	<i>Eco</i> RI
<i>Group 2 pollen allergen</i>	17,18	p3Polag2 F1-1	665	<i>Eco</i> RI
<i>Group 3 pollen allergen</i>	14,15	p5TriA3-1	653	<i>Eco</i> RI
<i>Group 4 pollen allergen</i>	1	p5TriA4 5.1	1106	<i>Eco</i> RI
<i>Group 5 pollen allergen</i>	4,5,22	p5Phlp5R2-3	785	<i>Nde</i> I + <i>Sac</i> II
<i>Group 7 pollen allergen</i>	20	p3G7F2-3	723	<i>Eco</i> RI
<i>Group 11 pollen allergen</i>	11,12	p3Phlp11F8-9	798	<i>Nde</i> I
<i>Pollen-specific protein</i>	13	p3PolspecF8-5	890	<i>Sac</i> II + <i>Nde</i> I
<i>Polygalacturonase</i>	2	p5PolygalaR2-7	~801	<i>Sma</i> I + <i>Sac</i> II
<i>Glucan endo-β-1,3-Glucanase</i>	6	p3GlucanF4-4	~1400	<i>Eco</i> RI
<i>Actin depolymerizing factor</i>	10	p3ADF4-5	783	<i>Eco</i> RI + <i>Pst</i> I
<i>Pollen profilin</i>	19	p5Prof3R4-2	288	<i>Bcl</i> I*
<i>Profilin</i>	21	p5ProfR2-1	871	<i>Eco</i> RI
<i>Cyclophilin</i>	8	p3Cyclo4	607	<i>Eco</i> RI
<i>Glutathione peroxidase</i>	9	p5Glutpx4	723	<i>Eco</i> RI
<i>Gluteradoxin</i>	16	p3Glut4H2	802	<i>Eco</i> RI

*PCR amplified product using Prof3-F4 + 5' RACE inner primer was digested with *Bcl* I to yield a 288 bp probe fragment (position 538-825, acc. No. JN578720).

Table S5. Homology of representative triticale cDNA clones and their deduced protein sequences with known sequences.

Spot No.	Protein	cDNA clone	Nucleotide sequences (gi, % identity)	Amino acid sequences
1	Group 4 pollen allergen	<i>TriA4F3R4-1</i>	<i>Triticum aestivum</i> : Tri a 4A (gi: 55859457, 100%), Tri a 4B (gi: 55859459, 96%), <i>Secale cereale</i> : Sec c 4 alleleA (gi: 55859453, 94%), Sec c 4B (gi: 55859455, 90%), <i>Hordeum vulgare</i> : mRNA for predicted protein (gi: 326530110, 92%; gi: 326500445, 91%); Hor v 4 (gi: 55859461, 92%).	<i>T. aestivum</i> : Tri a 4A (gi: 55859458, 100%), Tri a 4B (gi: 55859460, 94%), <i>S. cereale</i> : Sec c 4A (gi: 55859454, 92%), Sec c 4B (gi: 55859456, 88%), <i>H. vulgare</i> : Hor v 4 (gi: 55859462, 90%), predicted proteins (gi: 326530111, 326500446, 89%), <i>Phleum pratense</i> : Phl p 4 (gi: 82492267, 84%).
2	Polygalacturonase	<i>PGR2-7</i>	<i>H. vulgare</i> : mRNA for predicted protein (gi: 326513249, 92%), <i>P. pratense</i> : polygalacturonase (gi: 4826571, 87%), <i>Paspalum notatum</i> : group 13 grass pollen allergen (gi: 338930671, 338930673, 338930677, 338930675, 82%), <i>Zea mays</i> : Zea m 13 (gi: 89892726, 80%; gi: 162459707, 71.1%), polygalacturonase (gi: 288611, 78%), <i>Sorghum bicolor</i> : exopolygalacturonase precursor (gi: 242093569, 79%).	<i>H. vulgare</i> : predicted protein (gi: 326513250, 92%), <i>P. pratense</i> : polygalacturonase (gi: 4826572, 86%), <i>S. bicolor</i> : exopolygalacturonase precursor (gi: 242093570, 70%), <i>Z. mays</i> : polygalacturonase (gi: 288612, 70%), ze m 13 allergen (gi: 89892725, 70%).
3	Expansin B4	<i>ExpB4F5R3-2</i>	<i>T. aestivum</i> : expansin B4 (gi: 44894801, 99%; gi: 241984992, 93%), <i>H. vulgare</i> : mRNA for predicted protein (gi: 326501845, 92%), <i>Phalaris aquatica</i> : Pha a 1 (gi: 1246115, 87%), <i>Holcus lanatus</i> : Hol l l (gi: 1167835, 3860383, 87%), <i>P. pratense</i> : Phl p l (gi: 3901093, 473359, 85%), <i>Dactylis glomerata</i> : Dac g 1.01 (gi: 33149332, 85%), <i>Poa pratensis</i> : group 1 grass pollen allergen (gi: 4090264, 85%), <i>Lolium perenne</i> : Lol p l (gi: 19495, 85%).	<i>T. aestivum</i> : expansin B4 (gi: 44894802, 99%), <i>H. vulgare</i> : predicted protein (gi: 326501846, 88%), <i>H. lanatus</i> : expansin B4 (gi: 1171005, 80%), <i>D. glomerata</i> : expansin B4 (gi: 33149335, 79%), <i>L. perenne</i> : Lol p l (gi: 6599300, 80%; gi: 168316, 78%), <i>P. pratense</i> : Phl p l (gi: 3901094, 77%).
4	Group 5 pollen allergen	<i>Tris5R8-5</i>	<i>H. vulgare</i> : mRNA for predicted protein (gi: 326518113, 94%; gi: 326527678, 91%), Hor v 9 (gi: 459162, 1808986, 93%), <i>S. cereale</i> : Sec c 5 (gi: 332205750, 87%), <i>T. aestivum</i> : Hor v 9-like (gi: 40644791, 90%), mRNA (gi: 241983741, 88%), <i>P. pratense</i> : Phl p 5a (gi: 29500896, 84%; gi: 3309038, 3135500, 83%).	<i>H. vulgare</i> : predicted protein (gi: 326518114, 94%; gi: 326527679, 88%), Hor v 9 (gi: 1808987, 79%), <i>S. cereale</i> : Sec c 5 (gi: 332205751, 77%), <i>T. aestivum</i> : Hor v 9-like (gi: 40644792, 76%), Phl p 5-like (gi: 40644796, 59%), <i>P. pratensis</i> : group 9 pollen allergens (gi: 113561, 72%), <i>P. pratense</i> : Phl p 5 (gi: 3135499, 3135501, 398830, 73% ; gi: 3135503, 72% ; gi: 1684720, 3135497, 2851456, 71%), <i>L. perenne</i> : Lol p 5b (gi: 2498582, 67%).
5	Group 5 pollen allergen	See spot 4		

6	Glucan endo- β -1,3-Glucanase	<i>GlucanR3-4</i>	<i>T. aestivum</i>: glucan endo-β-1,3-glucanase (gi: 241985854, 115305906, 100%; gi: 115305908, 95%; gi: 109150355, 94%; gi: 115305905, 109150347, 84%; gi: 109150357, 83%; gi: 61657663, 241986800, 74%), <i>H. vulgare</i>: glucan endo-β-1,3-glucanase (gi: 29569879, 82%; gi: 326495211, 29569881, 81%), <i>S. cereale</i>: glucan endo-1,3-β-D-glucosidase (gi: 104161961, 76%).	<i>T. aestivum</i>: glucan endo-β-1,3-glucanase (gi: 115305907, 100%; gi: 109150356, 92%; gi: 115305909, 91%; gi: 109150350, 74%; gi: 109150348, 109150358, 71%), <i>H. vulgare</i>: glucan endo-1,3-beta-D-glucosidase (gi: 29569880, 29569882, 73%; gi: 326495212, 72%), <i>S. cereale</i>: glucan endo-1,3-β-D-glucosidase (gi: 104161960, 58%).
7	Expansin B1	<i>ExM2</i>	<i>T. aestivum</i>: expansin B1 (gi: 42601004, 99%), expansin B2 (gi: 42601006, 97%), expansin B5 (gi: 44894803, 99%), <i>H. vulgare</i>: mRNA sequence (gi: 326515629, 90%), <i>Oryza sativa</i>: beta expansin (gi: 115483269, 83%), pollen allergen mRNA (gi: 33338560, 83%), expansin B9 (gi: 8118436, 83%), <i>Z. mays</i>: mRNA (gi: 224035092, 80%), expansin B1 (gi: 14193760, 80%), <i>H. lanatus</i>: mRNA for Hol I I protein (gi: 1167835, 78%).	<i>T. aestivum</i>: expansin B1 (gi: 42601005, 99%), expansin B2 (gi: 42601007, 97%), and expansin B5 (gi: 44894804, 97%), <i>H. vulgare</i>: expansin like B1 (gi: 326515630, 85%), <i>O. sativa</i>: beta-expansin (gi: 115483270, 75%), <i>Paspalum notatum</i>: expansin A (gi: 168419914, 73%), <i>Z. mays</i>: expansin B9 (gi: 162460198, 70%), expansin B1 (gi: 115502388, 14193761, 69%).
8	Cyclophilin	<i>CycloF4R-1</i>	<i>T. aestivum</i>: cyclophilin A-2 (gi: 13925733, 100%), cyclophilin A (gi: 42493198, 100%), cyclophilin A-1 (gi: 13925730, 95%), cyclophilin A-3 (gi: 13925736, 95%), <i>Dasypyrum villosum</i>: cyclophilin 5 (gi: 156193316, 94%), cyclophilin 2 (gi: 156193310, 92%), cyclophilin 4 (gi: 156193314, 91%), cyclophilin 1 (gi: 156193308, 91%), cyclophilin 3 (gi: 156193312, 90%), <i>H. vulgare</i>: cDNA sequence (gi: 151426727, 92%).	<i>T. aestivum</i>: Cyclophilin A-2 (gi: 13925734, 100%), cyclophilin A-1 (gi: 13925731, 156193313, 99%), cyclophilin (gi: 154761388, 97%), <i>Dasypyrum villosum</i>: cyclophilin 3 (gi: 156193313, 98%), cyclophilin 1(gi: 156193309, 96%), cyclophilin 2 (gi: 156193311, 156193317, 96%), <i>S. bicolor</i>: cyclophilin (gi: 242060246, 88%).
9	Glutathione peroxidase	<i>Glutpx2</i>	<i>T. monococcum</i>: glutathione peroxidase-like sequence (gi: 34334011, 99%), <i>Claviceps purpurea</i>: glutathione peroxidase-like sequence (gi: 240853501, 95%), <i>Phyllostachys edulis</i>: glutathione peroxidase-like sequence (gi: 242387986, 242387742, 94%), <i>H. vulgare</i>: mRNA of a predicted protein (gi: 151418300, 326489307, 92%), glutathione peroxidase-like sequence (gi: 6179603, 92%).	<i>T. monococcum</i>: cytosolic glutathione peroxidase (gi: 34334012, 100%), <i>H. vulgare</i>: glutathione peroxidase-like protein (gi: 6179604, 326489308, 98%), <i>S. bicolor</i>: putative glutathione peroxidase (gi: 48374968, 95%), <i>Setaria italica</i>: glutathione peroxidase (gi: 44663004, 93%), <i>Z. mays</i>: glutathione peroxidase (gi: 162458182, 93%).
10	Actin Depolymerizing Factor	<i>ADF4-5</i>	<i>H. vulgare</i>: mRNA for predicted protein (gi: 326524891, 94%), <i>Z. mays</i>: actin depolymerizing factor (gi: 226503550, 90%; gi: 226493186, 88%), <i>S. bicolor</i>: hypothetical protein gene (gi: 242062999, 90%).	<i>H. vulgare</i>: actin depolymerizing factor (gi: 326524892, 97%), <i>O. sativa</i>: actin depolymerizing factor/cofilin-like protein (gi: 115447755, 95%), <i>Z. mays</i>: actin depolymerizing factor/cofilin-like protein (gi: 226503551, 94%), <i>S. bicolor</i>: actin depolymerizing factor (gi: 242063000, 94%).

11	Group 11 pollen allergen	<i>Tris11F8-9</i>	H. vulgare: mRNA for predicted protein (gi: 326518137, 96%), P. pratense: Phl p 11 (gi: 23452312, 91%), O. sativa: Phl p 11-like (gi: 115468515, 32982534, 85%).	H. vulgare: predicted protein (gi: 326518138, 93%), P. pratense: Phl p 11 (gi: 47606039, 85%), L. perenne: Lol p 11 (gi: 47605808, 86%), S. bicolor: hypothetical protein (gi: 242051507, 72%), Z. mays: Phl p 11 (gi: 226505364, 75%).
12	Group 11 pollen allergen	See spot 11		
13	Pollen-specific allergen	<i>PolspecF8-5</i>	T. aestivum: mRNA sequence (gi: 241984968, 99%), H. vulgare: mRNA for a predicted protein (gi: 326504813, 93%), O. sativa: pollen-specific protein C13 precursor (gi: 115458147, 79%).	H. vulgare: pollen Ole e I-related protein (gi: 326504814, 92%), O. sativa: pollen Ole e 1-related protein (gi: 116309466, 75%), pollen-specific protein (gi: 20310, 72%), Z. mays: pollen-specific protein C13 precursor (gi: 195622786, 70%).
14	Group 3 pollen allergen	<i>TrisA3-1</i>	T. aestivum: Tri a 3 (gi: 972512, 97%), H. vulgare: mRNA for a predicted protein (gi: 326529518, 85%), D. glomerata: Dac g 2 (gi: 4007039, 75%), C. dactylon: Cyn d 2 (gi: 4006977, 75%).	T. aestivum: Tri a 3 (gi: 972513, 98%), H. vulgare: predicted protein (gi: 326529519, 84%), P. pratense: Phl p 3 (gi: 39841264, 78%), D. glomerata: Dac g 3 (gi: 14423759, 73%), L. perenne: Lol p 3 (gi: 126387, 72%), Lol p 2-A (gi: 126387, 50%).
15	Group 3 pollen allergen	See spot 14		
16	Glutaredoxin	<i>Glut4H2</i>	T. aestivum: glutaredoxin (gi: 194295631, 95%; gi: 32401361, 94%), Z. mays: glutaredoxin (gi: 77817866, 94%), H. vulgare: mRNA for predicted protein (gi: 326489532, 91%), glutaredoxin (gi: 151419308, 91%), Deschampsia Antarctica: glutaredoxin (gi: 20141082, 79%).	T. aestivum: glutaredoxin (gi: 194295632, 94%; gi: 32401362, 95%), H. vulgare: glutaredoxin (gi: 326489533, 93%), S. bicolor: glutaredoxin-C6 (gi: 242073660, 81%), Z. mays: glutaredoxin (gi: 226533232, 77%).
17	Group 2 pollen allergen	<i>Polag2R4H-2</i>	H. vulgare: mRNA for predicted protein (gi: 326491018, 82%; gi: 326529518, 70%), T. aestivum: cDNA sequence (gi: 241985509, 81%), Tri a 2 (gi: 4007851, 69%), Tri a III (gi: 972512, 70%), L. perenne: Lol pII (gi: 431863, 74%), C. dactylon: Cyn d 2(gi: 4006977, 70%), P. pratensis: Poa p 2(gi: 4007654, 69%), H. lanatus: Hol I 2 (gi: 4007083, 68%), P. pratense: Phl p 3 (gi: 39841263, 71%), D. glomerata: Dac g III (gi: 1825458, 71%), L. perenne: Lol p 3 (gi: 27883900, 70%).	H. vulgare: predicted protein (gi: 326491019, 74%), L. perenne: Lol p 2-A (gi: 126386, 68%), D. glomerata: Dac g II (gi: 1093120, 67%), T. aestivum: Tri a III (gi: 972513, 51%).
18	Group 2 pollen allergen	See spot 17		

19	Pollen Profilin	<i>Prof3R4-2</i>	<i>P. pratense</i>: profilin 4 (gi: 2415701, 95%), profilin 2 (gi: 2415697, 94%), <i>T. aestivum</i>: unknown mRNA sequence (gi: 241987745, 93%), <i>H. vulgare</i>: mRNA for predicted protein (gi: 326522579, 88%).	<i>P. pratense</i>: profilin/Phl p 12 (gi: 3914432, 96%), profilin 1 (gi: 464471, 95%), profilin 3 (gi: 3914429, 94%), pollen profilin variant 4 (gi: 110644910, 93%), <i>H. vulgare</i>: profilin (gi: 326522580, 95%), <i>S. bicolor</i>: profilin (gi: 242091151, 83%), <i>Z. mays</i>: profilin (gi: 162461525, 82%), <i>Raphanus sativus</i>: profilin (gi: 158122094, 82%), <i>T. aestivum</i>: profilin (gi: 300807845, 79%).
20	Group 7 pollen allergen	<i>G7F2-3</i>	<i>O. sativa</i>: Phl p 7-like polcalcin (gi: 115477828, 93%), <i>S. bicolor</i>: group 7 allergen (gi: 242081808, 92%), <i>Z. mays</i>: mRNA sequence (gi: 195634662, 91%), <i>H. vulgare</i>: mRNA for a predicted protein (gi: 326527422, 91%), <i>P. pratense</i>: Phl p 7 (gi: 3367731, 86%).	<i>P. pratense</i>: putative calcium-binding Phl p 7 pollen allergen (gi: 14423846, 99%), <i>H. vulgare</i>: predicted calcium-binding protein (gi: 326527423, 99%), <i>O. sativa</i>: putative calcium-binding Phl p 7 pollen allergen (gi: 115477829, 99%), <i>C. dactylon</i>: putative calcium-binding Phl p 7 pollen allergen (gi: 1864024, 94%).
21	Profilin	<i>ProfR2-2</i>	<i>T. aestivum</i>: profilin (gi: 190684060, 300807844, 98%; gi: 1008444, 87%), <i>H. vulgare</i>: mRNA sequence (gi: 151426039, 151419502, 93%), mRNA for predicted protein (gi: 326501767, 93%), profilin (gi: 1229168, 96%), <i>Z. mays</i>: mRNA sequence (gi: 77817424, 96%).	<i>H. vulgare</i>: profilin (gi: 326501768, 100%; gi: 1709779, 98%), <i>T. aestivum</i>: profilin (gi: 300807845, 99%; gi: 190684061, 96%), <i>P. pratense</i>: profilin (gi: 3914432, 84%).
22	Group 5 pollen allergen	See spot 4		