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Isoforms of group 1 allergens from a tropical/subtropical para grass (*Urochloa mutica*) display different levels of IgE reactivity and cross-reactivity

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Key words

para grass pollen; beta-expansins; isoallergens; Uro m 1; recombinant group-1 allergen

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Summary

Group 1 grass pollen allergens, or beta-expansins, are the most important major allergens from tropical/subtropical grasses. This study aimed to investigate the sequence similarity and immunoreactivity of group 1 allergens from para grass (Urochloa mutica). Three isoforms (Uro m 1.01, Uro m 1.02, and Uro m 1.03) were cloned from cDNA of para grass pollen. The acidic-neutral isoforms rUro m 1.01 and rUro m 1.02 could effectively inhibited beta-expansins in pollen extract of Bermuda and Johnson grasses, suggesting that these isoforms could be major cross-reacting allergens among these grasses. In contrast, the basic isoform rUro m 1.03 had limited IgE reactivity. Thus, group 1 allergens both acidic-neutral and basic isoforms could have markedly different IgE reactivity.

Introduction

Grass pollen (GP) is among the most significant sources of outdoor airborne allergens worldwide. Nonetheless, information about tropical / subtropical GP allergens is relatively limited despite the high diversity of grasses in these regions. Allergenicity and cross-reactivity of GP are often presumed from prior knowledge in the temperate regions, ignoring the genuine sources of sensitization and the complexity / redundancy of multiple allergens and their isoforms. This, combined with the inadequate patient profiles from large scale studies, hinders the expansion of our understanding of tropical / subtropical GP allergy. Hence, identification of local tropical / subtropical grass species and their GP major allergens should be encouraged.

The dominating grass species in the tropical / subtropical regions mostly belong to the subfamily Chloridoideae (e.g. *Cy-*

nodon dactylon, Bermuda grass) and Panicoideae (e.g. *Paspalum notatum*, Bahia grass; *Sorghum halepense*, Johnson grass; and *Zea mays*, maize), while Pooideae grasses (e.g. *Lolium perenne*, rye grass; *Phleum pratense*, Timothy grass; *Dactylis glomerata*, orchard grass; *Phalaris aquatica*, Canary grass; *Poa pratensis*, Kentucky blue grass; and *Anthoxanthum odoratum*, sweet vernal grass) are largely absent in these regions. The main difference relevant to allergenicity is that the group 5 GP allergens are not present in Chloridoideae and Panicoideae GP. On the other hand, the group 1 GP allergens have been identified as the most prevalent and potent allergens in all grass species (1,2). Group 1 GP allergens have been categorized as a subclass of the beta-expansin family (3). These glycoproteins are highly expressed in GP and secreted at pollen walls to assist pollen-tube penetration (4). Like other expansins, beta-expansins loosen plant cell walls by disrupting hydrogen bonds between cellulose microfibrils and cross-linking glycans in plant cell wall (5).

Group 1 GP allergens have been reported as major allergens in several grass species. The most notable members are Cyn d 1 in Bermuda grass, Sor h 1 in Johnson grass, Zea m 1 in maize, Pas n 1 in Bahia grass, Ory s 1 in rice, Phl p 1 in Timothy grass, and Lol p 1 in rye grass (6-12). Group 1 allergens in Pooideae grasses, have highly conserved amino acid sequences (85 - 95% identity) and were shown to have comparable levels of allergenicity among species (13). Sequence homology group 1 allergens from different subfamilies such as Phl p 1 (Pooideae) and Cyn d 1 (Chloridoideae) was considerably lower (67 - 70% identity) and these allergens were shown to have incomplete inhibition (14). Therefore, it was largely assumed that the degree of similarity and, consequently, cross-reactivity of group 1 allergens largely corresponded to the taxonomic relationship of the grass species. However, as more genomic, transcriptomic, and proteomic information became available, it was clear that several genes encoding beta-expansins could be present in a given genome, giving rise to several isoforms (isoallergens) from a single species. For example, two isoforms with 60% sequence identity have been reported in Zea m 1 and Sor h 1 (7,8,15). Different amino acid sequences might affect IgE binding and allergenicity, but direct comparison between isoforms from a single species has been scarce.

Para grass or *Urochloa mutica* (formerly *Brachiaria mutica*) had been reported as one of the top allergenic grass species in Thailand (16,17). Of the 2,383 AR patients attending the ENT Allergy Clinic at Siriraj Hospital, Bangkok, between 2005 - 2014, 53.2% had positive skin prick test reaction to para grass pollen (PGP) extract (17). However, allergenicity of PGP has rarely been reported in other countries. This grass species is native to Africa, but had been introduced to tropical and subtropical regions of the world as fodder grass. It is currently considered one of the worst grass species in several regions, including the United States, Central America, Australia, and Asia (18). With a wide range of tolerable climates and soil conditions and its aggressively invasive nature, this species has been rapidly increasing. Due to the large distribution area of para grass, it is possible that the GP is an important allergen source and the incidence of sensitization could be rising in tropical/subtropical regions.

In our previous study, beta-expansin had been reported as a major cross-reactive allergen among Bermuda, Johnson, and para grass (19). In this study, we aimed to clone the different isoforms of beta-expansins from PGP, characterize their IgE reactivity and determine their ability to inhibit IgE binding to crude extracts of other grasses. This study provided information about the major allergenic components of an unreported allergenic grass species, and compared the IgE-reactivity of the different isoforms of group 1 GP allergens. The knowledge obtained from this study may be useful for improving diagnosis and immunotherapy for tropical/subtropical GP allergy.

Materials and methods

Serum samples

Serum samples were obtained as a part of the "Development of Siriraj Pollen Allergen Vaccine (SPAV)" project, in accordance with the approved ethics for research in humans by the Siriraj Institutional Review Board, Mahidol University, Bangkok, Thailand (SiEc100/2012). Forty patients with AR history were skin prick tested (SPT), and seventeen of them were sensitized to PGP. Six sera with IgE reactivity to Uro m 1 were included in this study based on the criteria as described previously (19) (**table A**).

Pollen protein extraction and SDS-PAGE

GP were collected from various natural sites within Bangkok metropolitan area and extracted in phosphate buffer saline (PBS) (19). PG extracts were separated by 12% sodium dodecyl

Table A *- Demographic of individual sera from allergic rhinitis patients of this study.*

no.	sex	age	SPT (mean wheal diameter, mm)			sIgE (kUA/L)	
			BGP	JGP	PGP	BGP	JGP
9	F	46	9	5	3	30.3	34.7
12	M	52	4	3.5	3	0.16	0.05
17	M	30	4	4	2	4.16	5.19
29	M	21	5	3	2	4.02	3.05
33	M	10	8.5	4.5	3.5	> 100	85.3
36	M	18	4	5	4.5	9.1	13.6

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For visualization, SDS-PAGE gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 (Merck, USA).

Cloning of cDNA encoding beta-expansins from PGP

Total RNA was extracted from PGP using TRIzol™ Reagent kit (Invitrogen, USA). RNA was converted into cDNA using oligo-dT primer and iScript reverse transcriptase (Bio-Rad, USA). Coding sequences of beta-expansins were amplified by PCR using Platinum® Taq DNA Polymerase High Fidelity (Thermo Fisher Scientific, USA). Three beta-expansins cDNAs were amplified by primers designed from group 1 GP allergens or beta-expansins from related grasses e.g. Cyn d 1, Zea m 1, Sor h 1, beta-expansins of *Setaria italica* (**table I**). The gel purified PCR products were elongated A-tail, and then inserted into pGEM®-T easy vector (Promega, USA). The recombinant plasmids were transformed into *Escherichia coli* DH5α, and then submitted to sequence by the AIT biotech Pte Ltd, Singapore.
The obtained sequences were manually edited and subjected to homology search using BLAST against the Genbank database. The nucleotide sequences were deposited in GenBank database: Uro m 1.01 (MH820172), Uro m 1.02 (MH820171), and Uro m 1.03 (MH820173). Deduced amino acid sequences were analyzed by ExPaSy proteomic tools such as ProtParam (20), ScanProsite (21), and CFSSP (22). N-terminus signal sequence and expansin domains were characterized by SignalP (23) and ScanProsite (21). The conserved regions of Uro m 1 and the 67-100% homologous sequences were analyzed by ConSurf (24). Nucleotides and amino acid sequences were compared with grass pollen allergens reported by the International Union of Immunological Societies (IUIS) Sub-committee for Allergen Nomenclature. Multiple sequence alignment and percent identity matrix were performed using Clustal Omega (25). A phylogenetic tree was reconstructed based on amino acid sequences. The evolutionary analyses were conducted in MEGA7 (26) using the Neighbor-Joining method (27) and the evolutionary distances were computed using the Poisson correction method (28).

Expression and purification of recombinant proteins

PCR products of beta-expansins contained endonuclease enzyme recognition sites were amplified using primers listed in **table I**. The PCR products were double digested for Uro m 1.01 or Uro m 1.02 and Uro m 1.03 constructions using Eco RI/Xho I or Nde I/Hind III (NEB, USA), respectively, then gel-purification and sub-cloning of PCR pieces into the expression vector pET-28a(+) (Novagen, Germany). The DNA sequence was confirmed by sequencing of both strands. Expression vectors were transformed into expressing host *E. coli* BL21 (DE3) pLysS cells.
The recombinant protein expression was induced by addition isopropyl β-D-thiogalactopyranoside (IPTG) (Amresco, USA and Canada) at 37 °C. Bacterial cells were harvested and the

Table I *- Primer list for cDNA cloning and expression vector construction of three Uro m 1 isoforms.*

Name	Sequence1	Ta (°C)	Product (bp)
	cDNA cloning		
U1F1	5'-GCC AAG CTT GAA TTC GAA CAG GCT ATG CTC GCG-3'	55	Uro m 1.01 (829 bp)
U1R1	5'-GCC AAG CTT CTC GAG TCA GAA CTG GAT CTT GGA CTT GTA GAC-3'		
U2F1	5'-GAC GGC AAG TGG CTG GAC-3'	57	Uro m 1.02 (695 bp)
U2R1	5'-GGA ATT AGA ACT GGA GCT TGG AG-3'		
U3F1	5'-GCA ACA GCC ACA CAC AAC AAC-3	55	Uro m 1.03 (984 bp)
U3R1	5'-GTG AGC CCG GAT TAC AGA TTA G-3		
	expression vector construction		
U1F2	5'-GCC AAG CTT GAA TTC GAA CAG GCT ATG CTC GCG-3' (Eco RI)	60	Uro m 1.01 (829 bp)
U1R2	5'-GCC AAG CTT CTC GAG TCA GAA CTG GAT CTT GGA CTT GTA GAC-3' (Xho I)		
U2F2	5'-CTA TTA CAT ATG TGG CTG GAC GCC AAG GCG ACG TG-3' (Nde I)	62	Uro m 1.02 (708 bp)
U2R2	5'-CGT GCG AAG CTT GGA ATT AGA ACT GGA GC-3' (Hind III)		
U3F2	5'-GGC AGC CAT ATG TGG CTC CCC GCC AGG GCC AC-3' (Nde I)	60	Uro m 1.03 (776 bp)
U3R2	5'-GTG AGC CCG GAT TAC AGA TTA G-3 (Eco RI)		

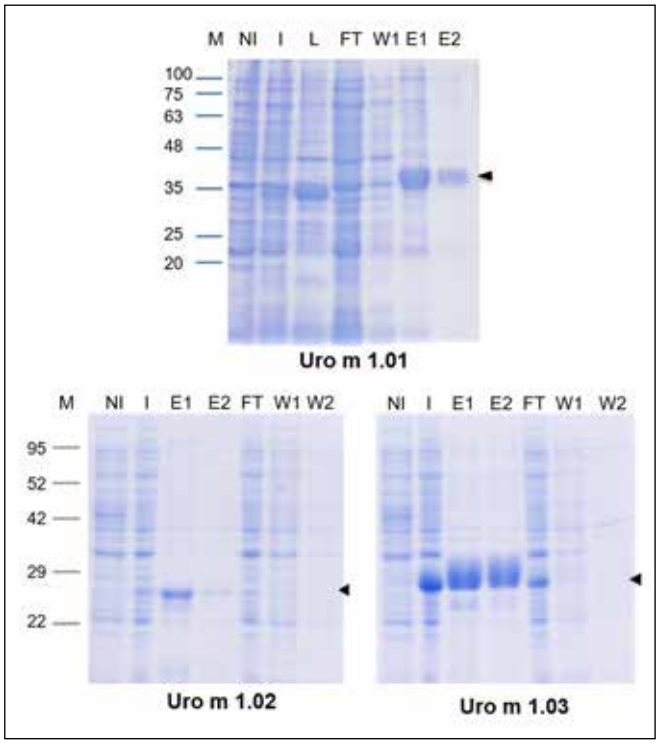
[1]Underlined letters indicate endonuclease recognition sites. Restriction enzymes specific to each primer are presented in parentheses.

recombinant proteins were found mostly in the insoluble fractions of bacterial extracts. Therefore, recombinants having a 6-His tag at the N-terminus were purified from the inclusion body in cell pellets under denaturing condition using Ni-NTA resins (Novex®, USA). The recombinant proteins had 71%, 89%, and 85% purity for rUro m 1.01, rUro m 1.02, and rUro m 1.03, respectively, analysed by measuring intensity of eluted protein bands from SDS-PAGE gel using ImageJ (**table V**).

Immunoblotting and inhibition

Protein extracts were separated by electrophoresis in 12% SDS-polyacrylamide gels using 0.5 and 5 µg of recombinants and GP proteins, respectively, per well. The separated proteins were electro-transferred from gels to nitrocellulose membranes using a Mini Tran-Blot® Electrophoretic Transfer Cell (Bio-Rad, USA). The membrane was incubated overnight at 4°C with patient's sera diluted at 1/100 - 1/5,000 in phosphate buffer saline (PBS) PBS containing 3% (w/v) skim milk. Washed membrane then incubated with a 1:10,000 diluted HRP-labeled mouse IgG anti-human IgE antibodies (KLP, USA) for 2 hr at room temperature. Bound IgEs were detected with Immobilon™ Western Chemiluminescent HRP substrate (Millipore, Germany) and emitted signal was captured by x-ray films.
The inhibition assays were performed similarly, except that the diluted sera were pre-incubated with 50 ng/µl recombinant proteins (19). The recombinant D7 protein from mosquito *Aedes aegypti* was used as a negative control. Recombinant D7 protein was expressed and purified in a system comparable to that of recombinant Uro m 1.

Table V *- Purification of 6X-His tag proteins using Ni-NTA resins (Novex®, USA) under denaturing conditions. The arrowheads indicated recombinant protein bands. NI, Un-induced cells; I, induced cells; L, induced cell lysate; E1-E2, recombinant elution fraction; FT, flow-through fraction; W1-W2, wash fraction.*



Enzyme-linked immunosorbent assay (ELISA) and inhibition ELISA

GP proteins were diluted with PBS and coated on ELISA plates at 1 µg/well at 4°C overnight. The coated plate was washed and incubated with 1/4 - 1/64 diluted sera in PBS containing 3% (w/v) skim milk. After sera incubation, bound IgEs were detected by incubation with 1/5,000 diluted HRP-labeled mouse IgG anti-human IgE anti-human antibodies. 3,3'5,5-tetramethylbenzidine (TMB) substrate (KPL Inc., USA) was added to each well and the color reaction stopped using 1 N HCl. Light absorbance was measured at a wavelength of 450 nm. All determinations were performed in duplicate.
The inhibition assay was performed in a similar manner. The serum from each individual was tested at one dilution at 1/4 - 1/64, which was determined from a preliminary experiment to show sufficient IgE binding and inhibition. The inhibitors were tested in the range of 15-200 ng/µl recombinant proteins. The experiment was performed in duplicate (19).

Results

Sequences and characteristics of (Uro m 1) beta expansin isoforms from Para grass

Molecular cloning of beta-expansins from PGP was performed based on conserved regions to reported beta-expansins from other grass species. Several clones were obtained and sequenced and were classified into three separate isoforms: Uro m 1.01, Uro m 1.02, and Uro m 1.03. Information about these three isoforms were deposited in the IUIS Sub-committee for Allergen Nomenclature database. ORF length, homologous sequence, and sequence variation of all identified clones of three Uro m 1 are presented in **table II**. The highest sequence identity among clones was seen with Uro m 1.01. Interestingly, these Uro m 1.01 clones were nearly identical to Cyn d 1 isoforms 2 and 4 (99 - 100%), differing only in one or two nucleotides. Uro m 1.02 and Uro m 1.03 clones had higher clone variation than Uro m 1.01. The three isoforms shared only 70-79% nucleotide sequence identity.

Table II *- Sequence variation of Uro m 1 peptidic epitopes predicted from well-known allergens*

Allergen	Position	% Epitope conservancy[1]	Epitope	Sequence
			Pas n 1 epitope	
Pas n 1	123-142	-	T cell	IAPYHFDLSGKAFGAMAKPG
Uro m 1.01	119-138	90		IA**A**YHFDLSGKAFGAMAK**K**G
Uro m 1.02	86-105	80		IA**A**YHFDL**A**G**T**AFGAMAK**K**G
Uro m 1.03	124-143	95		IAPYHFDLSGKAFGA**L**AKPG
Pas n 1	177-196	-	T cell	GSNPNYLAMLVKFVADDGDI
Uro m 1.01	173-192	75		GSNPNYLA**L**LVK**YAAG**DGN**I**
Uro m 1.02	140-159	65		G**C**NPNY**FALLIKYAAG**DGDI
Uro m 1.03	178-197	85		**AC**NPNYLA**V**LVKFVADDGDI
Pas n 1	240-259	-	T cell	GKKVIAQDVIPVNWKPDTVY
Uro m 1.01	237-256	65		G**GH**V**EQE**DVIP**ED**WKPDTVY
Uro m 1.02	204-223	50		G**TTLVQD**DA**I**P**EG**WK**A**DTVY
Uro m 1.03	242-261	65		GKK**LVAN**DVIP**A**NWK**AN**T**A**Y
			Cyn d 1 epitope	
Cyn d 1	109-128	-	T cell	SGKAFGAMAKKGQEDKLRKA
Uro m 1.01	127-14	95		SGKAFGAMAKKG**E**EDKLRKA
Uro m 1.02	94-113	80		**A**G**T**AFGAMAKKG**EEE**KLRKA
Uro m 1.03	132-151	75		SGKAFGA**L**AK**P**G**LN**DKLR**H**A
Cyn d 1	181-209	-	T cell	PKDSDEFIPMKSSWGAIWRIDPKKPLKGP
Uro m 1.01	199-227	83		**S**K**G**SDEF**L**PMK**Q**SWGAIWRIDP**P**KPLKGP
Uro m 1.02	166-194	72		**E**K**GSE**EFIP**LKH**SWGAIWRID**SP**KP**I**KGP
Uro m 1.03	204-232	66		**EKASAEWK**PMK**L**SWGAIWR**V**D**TP**K**A**LKGP
Cyn d 1	217-241	-	T cell	EGGAHLVQDDVIPANWKPDTVYTSK
Uro m 1.01	235-259	68		E**SGGH**V**EQE**DVIP**ED**WKPDTVY**K**SK
Uro m 1.02	202-226	76		EGG**TT**LVQDD**A**IP**EG**WK**A**DTVYTSK
Uro m 1.03	240-264	60		E**SGKK**LV**AN**DVIPANWK**AN**T**AYPSN**
Cyn d 1	88-97	-	IgE and IgG_4	CGSCYEIKCK
Uro m 1.01	88-97	100		CGSCYEIKCK
Uro m 1.02	55-64	90		CGSCYEIKC**D**
Uro m 1.03	93-102	90		CGSCYEI**R**CK
Cyn d 1	119-128	-	IgE	IAAYHFDLSG
Uro m 1.01	119-128	100		IAAYHFDLSG
Uro m 1.02	86-95	90		IAAYHFDL**A**G
Uro m 1.03	124-133	90		IA**P**YHFDLSG
Cyn d 1	162-171	-	IgG_4	SGTKITFHIE
Uro m 1.1	162-171	70		S**D**TKI**A**FH**V**E
Uro m 1.2	129-138	60		**AN**TKI**A**FH**V**E
Uro m 1.3	167-176	60		**GGQ**KI**V**FH**V**E
Cyn d 1	177-185	-	IgE and IgG_4	HYLALLVKY
Uro m 1.01	177-185	88		**N**YLALLVKY
Uro m 1.02	144-152	66		**NYF**ALL**I**KY
Uro m 1.03	182-190	66		**N**YLA**V**LVK**F**
Cyn d 1	190-199	-	IgE and IgG_4	GNIVSVDIKS
Uro m 1.01	190-199	100		GNIVSVDIKS
Uro m 1.02	157-166	70		G**D**IV**A**VDIK**E**
Uro m 1.03	195-204	40		G**D**IV**NMEL**K**E**
Cyn d 1	209-218	-	IgG_4 binding	KSSWGAIWRI
Uro m 1.01	209-218	90		K**Q**SWGAIWRI
Uro m 1.02	176-185	90		K**H**SWGAIWRI
Uro m 1.03	214-223	80		K**L**SWGAIWR**V**

[1]Epitope analysis tool obtained from IEDB.org was epitope conservancy analysis (38).
Nucleotide sequences from cDNA clones of different Uro m 1 isoforms.

Deduced amino acid sequences and their predicted features

The full-length mRNA transcripts of Uro m 1.01 and Uro m 1.03 could be translated to 262 and 267 amino acids, respectively, while the partial mRNA region of Uro m 1.02 coded for 229 amino acids (**table III**). The deduced amino acid sequence alignments of Uro m 1 are presented in **figure 1**. All isoforms of Uro m 1 contained conserved features of expansins such as signal peptide, glycosylation site, HFD motifs and cysteine (C) residues in expansin family-45 endoglucanase-like (expansin-like EG45) domain, and tryptophan (W) residue in expansin cellulose-binding like (expansin-like CBD) domain. The high sequence homology to other group 1 grass pollen allergens and the presence of conserved amino acid residues suggest that these sequences encode beta-expansins group 1 grass pollen allergens of Para grass.

Based on the amino acid sequences, Uro m 1.01 and Uro m 1.02 were predicted to be acidic glycoproteins, while Uro m 1.03 was a basic glycoprotein with the approximate molecular mass of 28 kDa. Each Uro m 1 could harbour three disulfide bridges in expansin-like EG45 domain, which is the putative catalytic region. The percentage of alpha-helix forming regions is higher than beta-sheet forming regions in all Uro m 1. Based on the homologous sequence comparison using ConSurf algorithm (24), most conserved amino acid regions of Uro m 1 are located in the sites important for protein function rather than structure. So far, the only beta-expansins with characterized IgE and IgG4 antibody-binding epitopes/T cell epitope are Cyn d 1 and Pas n 1 (29, 30, 31). The corresponding epitopes were found within the three isoforms of Uro m 1, with some amino acid changes (**table II**). These differences might confer the unique and cross-reactive IgE-reactivity of Uro m 1 isoforms. Details of all Uro m 1 peptide sequences are presented in **table IV**.

Homology to previously characterized grass group 1 allergens

To determine the similarity between Uro m 1 isoforms and previously reported beta-expansins, the beta-expansin sequences with high similarity to Uro m 1 were obtained from GenBank and used for clustering analysis with Neighbor-Joining method. The resulting dendrogram is presented in **figure 2**. As expected, grass group 1 allergens are divided into two classes (A and B) (8, 15). Class A allergens can be further divided into four sub-groups, largely corresponding to the grass sub-family: (A-I) Pooideae allergens (e.g. Lol p 1, Phl p 1), (A-II) Ehrhartoideae allergen (Ory s 1), (A-III) Panicoideae allergens (e.g. Sor h 1, Zea m 1), and (A-IV) Chloridoideae allergens (Cyn d 1). Class B allergens were separated into two sub-groups: (B-I) Ehrhartoideae allergen (Ory s 1), and (B-II) Panicoideae allergens (e.g. Pas n 1, Sor h 1). Importantly, Uro m 1.01, Uro m 1.02 and Uro m 1.03 were clustered into different classes and subgroups (A-IV, A-III, and B-II, respectively). Other beta-expansins from a single plant species that were clustered into separate groups are Ory s 1, Sor h 1, and Zea m 1. In comparison with beta-expansins found in rice and maize genome Uro m 1.01 was most similar to OsEXPB1 (73%), ZmEXPB10 (71%), and ZmEX-

Table III - *Nucleotide sequences from cDNA clones of different Uro m 1 isoforms.*

Gene	No. of clones	Seq. identity among clones	Percentage of allergen identity (%)1	Sequence length
Uro m 1.01	3	99.8 - 99.9%	Cyn d 1 AF177380.1 (100%) Cyn d 1 AF177378.1 (99%) Cyn d 1 AF177379.1 (96%) Cyn d 1 AF177030.1 (93%) Cyn d 1 AF159703.2 (93%) Cyn d 1 S83343.1 (87%)	789 bp (262 aa)
Uro m 1.02 (partial)	4	87.9 - 100%	Sor h 1 KF887425.1 (83%) Zea m 1 DQ421827.1 (81%) Zea m 1 DQ421828.1 (80%) Cyn d 1 AF177030.1 (80%) Cyn d 1 AF159703.2 (80%) Cyn d 1 AF177380.1 (79%)	690 bp (229 aa)
Uro m 1.03	5	95.2 - 100%	Pas n 1 EU327342.1 (87%) Zea m 1 NM001111739.1 (86%) Zea m 1 L14271.1 (85%) Sor h 1 KF887426.1 (85%) Ory s 1 AF220610.1 (81%) Cyn d 1 AF177380.1 (70%)	804 bp (267 aa)

[1]Percentage of sequence identity was calculated to compare the sequence similarity between well-known allergens and Uro m 1. The NCBI accession numbers are presented following allergens' name. Percentages of sequence identities were presented in parentheses.

Figure 1 *- Alignment of deduced amino acid sequences among three Uro m 1. Opened, dark grey, and light grey boxes indicate N-terminus signal sequence, expansin family-45 endoglucanase-like (expansin-like EG45) domain, and expansin cellulose-binding like (expansin-like CBD) domain, respectively, predicted by SignalP (23) and ScanProsite (21). Bold letters indicate the highly conserved amino acid residues of each Uro m 1 and the 67-100% homologous sequences using ConSurf (24).*

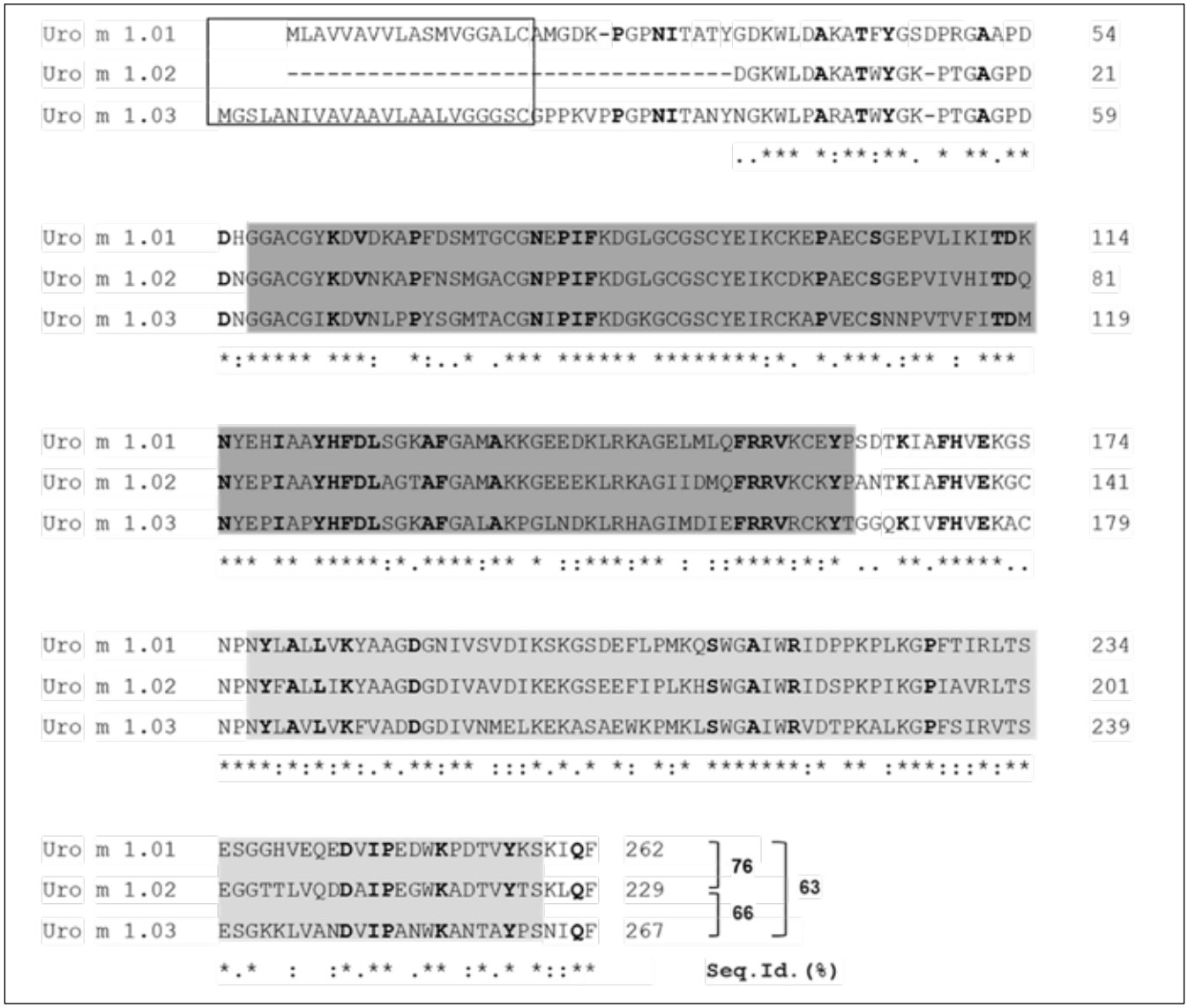
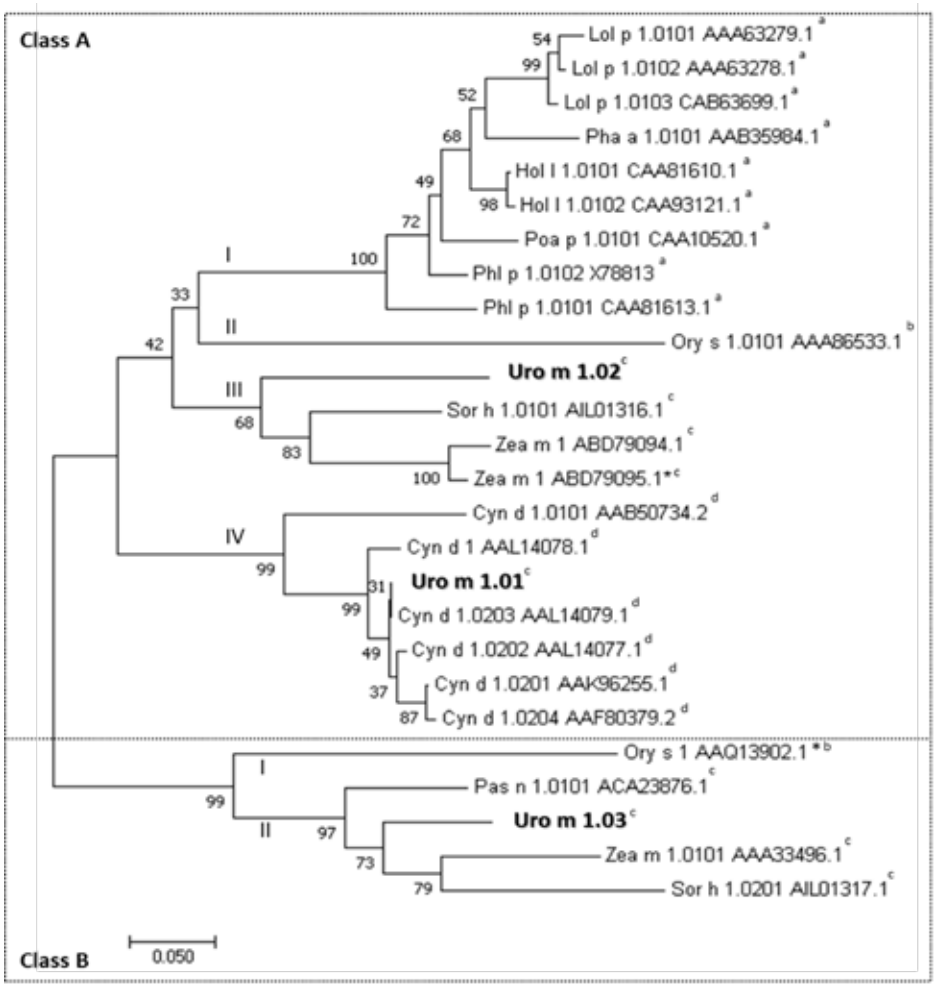


Table IV *-Amino acid sequence analysis using proteomic tools[1].*

Name	Molecular mass (kDa)	PI	No. and positions of disulfide bridges2/	Beta sheet/alpha helix (% / %)
Uro m 1.01	28.4	5.8	aa. 60-88, 91-158, and 96 - 102	51.5 / 66.8
Uro m 1.02 (partial)	24.8	6.5	aa. 27-55, 58-125, and 63 - 69	45.4 / 70.7
Uro m 1.03	28.6	9.1	aa. 65-93, 96-163, and 101 - 107	52.8 / 60.7

[1]Proteomic tools analysed Uro m 1 sequence were obtained from ExPASy.org such as ProtParam (Gasteiger et al. 2005), ScanProsite (de Castro et al. 2006), and CFSSP (Chou and Fasman 1974); [2]The numbers in parentheses indicate the amino acid regions forming a disulfide bridge.

Figure 2 - *The phylogenetic tree of group 1 GP allergens. The evolutionary analyses were conducted in MEGA7 (26) using the Neighbor-Joining method (27) and the evolutionary distances were computed using the Poisson correction method (28). Bootstrap values are shown at each node. Allergen sequences were retrieved from the WHO/IUIS Sub-Committee for Allergen Nomenclature database and the homolog proteins from Blastp analysis. The GenBank accession numbers are presented after allergen names. Superscript letters indicate grass subfamily (a, Pooideae; b, Ehrhartoideae; c, Panicoideae; d, Chloridoideae) and * indicates unpublished reference.*



PB11 (71%). Uro m 1.02 was similar to ZmEXPB11 (79%) and OsEXPB1 (77%), while Uro m1.03 was a close homolog of ZmEXPB1 (85%) and OsEXPB9 (80%).

Variation of IgE reactivity among different isoforms of rUro m 1

The three isoforms of Uro m 1 were cloned and expressed as recombinant proteins using the *E. coli* expression system. An immunoblot of purified recombinant Uro m 1 is shown in **figure 3**. Uro m 1.01 and Uro m 1.02 could interact with IgE in sera from all six atopic donors with PGP sensitization chosen for this study. On the other hand, there was no detectable IgE reactivity to Uro m 1.03 in sera from four patients, and only a relatively weak signal could be detected with serum number 33. No IgE reactivity to the 30 kDa protein in the pollen crude extract or recombinants was detected when sera of negative control individuals were used. These data showed that the three isoforms of rUro m 1 had different levels of IgE reactivity. Moreover, more than one isoform of beta-expansins could contribute, albeit not equally, to the overall IgE reactivity, and possibly to the allergic symptoms. Because Uro m 1.03, the class B beta-expansin, only showed weak IgE binding, this isoform was not used in further experiments.

Recombinant Uro m 1 isoforms with specific and dose-dependent IgE-reactivity

To confirm the specificity and IgE-binding properties of rUro m 1.01 and rUro m 1.02, we conducted immunoblot inhibition experiments. As shown in **figure 4a**, the IgE-binding signals of the 30 kDa proteins were largely reduced by serum pre-incubations with rUro m 1.01 and rUro m 1.02. The inhibition was not observed when recombinant D7 protein, a major allergen from mosquito, was used for pre-incubation, confirming that this inhibition was specific to rUro m 1. In ELISA inhibition, both rUro m 1.01 and rUro m 1.02 showed the inhibitory effects in a dose-dependent manner with all patient sera (**figure 4b**).

The inhibition by Uro m 1.01 calculated from log-concentration scale was relatively more effective than that of Uro m 1.02 at IC_{50} of 38.2 and 52.5 ng/µl, respectively. Of note, we could obtain the highest levels of inhibition at 70.1 ± 3.7% for Uro m 1.01 and 79.6 ± 9.5% for Uro m 1.02, which were not the maximum levels possible. Higher level of inhibition could not

Figure 3 *- Profiles of IgE bound proteins from PGP and rUro m 1. The arrows and arrowheads indicate the IgE-binding proteins of PGP and rUro m 1, respectively. Lane 1: PGP proteins; Lane 2: Uro m 1.01; Lane 3: Uro m 1.02; Lane 4: Uro m 1.03; P9-P36: Sera of PGP sensitized donors; N: Serum of non-sensitized donor.*

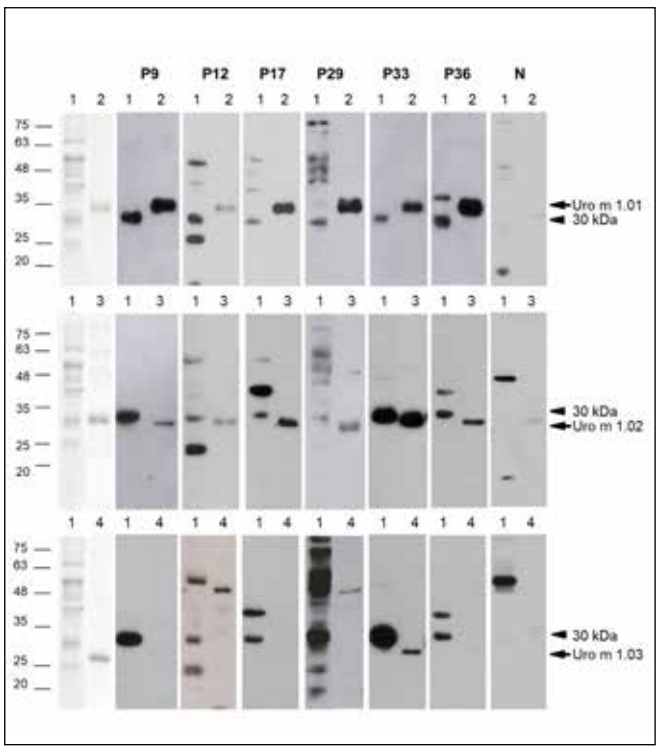
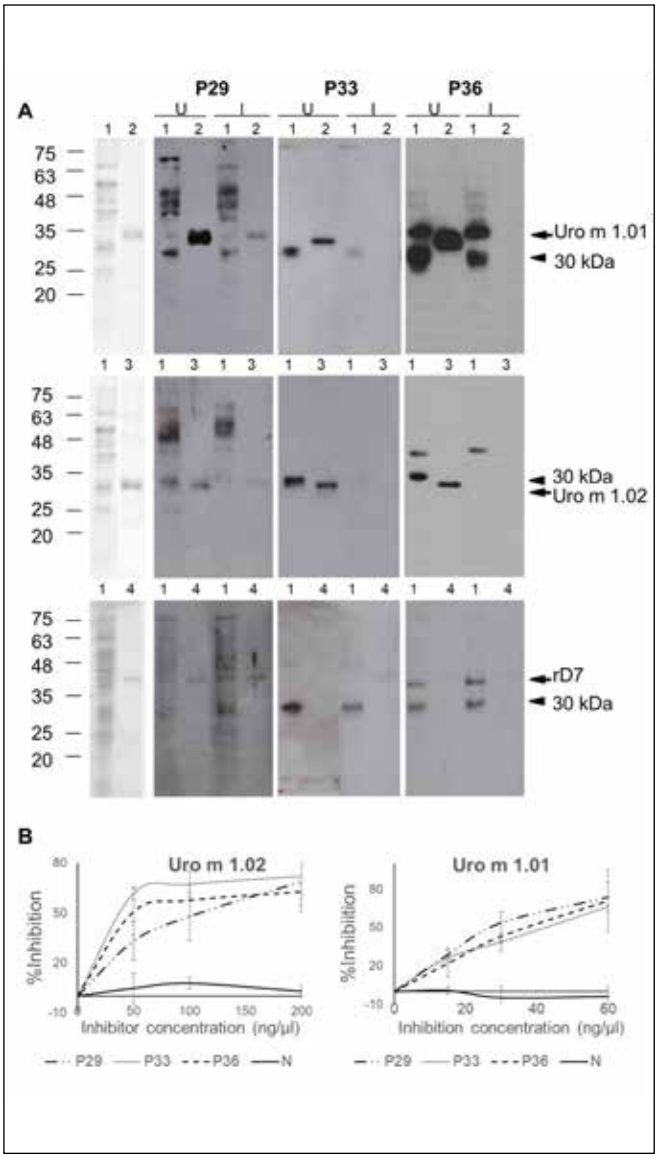


Figure 4 *- Inhibition of specific IgE binding to PGP with rUro m 1. The immunoblot inhibition of IgE-binding was performed using recombinant pre-incubated sera (I) in comparison with buffer pre-incubated sera (U) (A). The recombinant D7 protein from mosquito Aedes aegypti was used as a negative control. The arrows and arrowheads indicate the IgE-binding proteins of PGP and recombinant proteins, respectively. Percentage inhibition of specific IgE binding to PGP with rUro m 1 were detected in ELISA analysis (B). The data were performed in duplicate and presented as mean±SD. Lane 1: PGP proteins; Lane 2: Recombinant Uro m 1.01; Lane 3: Recombinant Uro m 1.02; Lane 4: Recombinant D7; P29-P36, Sera of PGP sensitized donors; N: Serum of non-sensitized donor.*

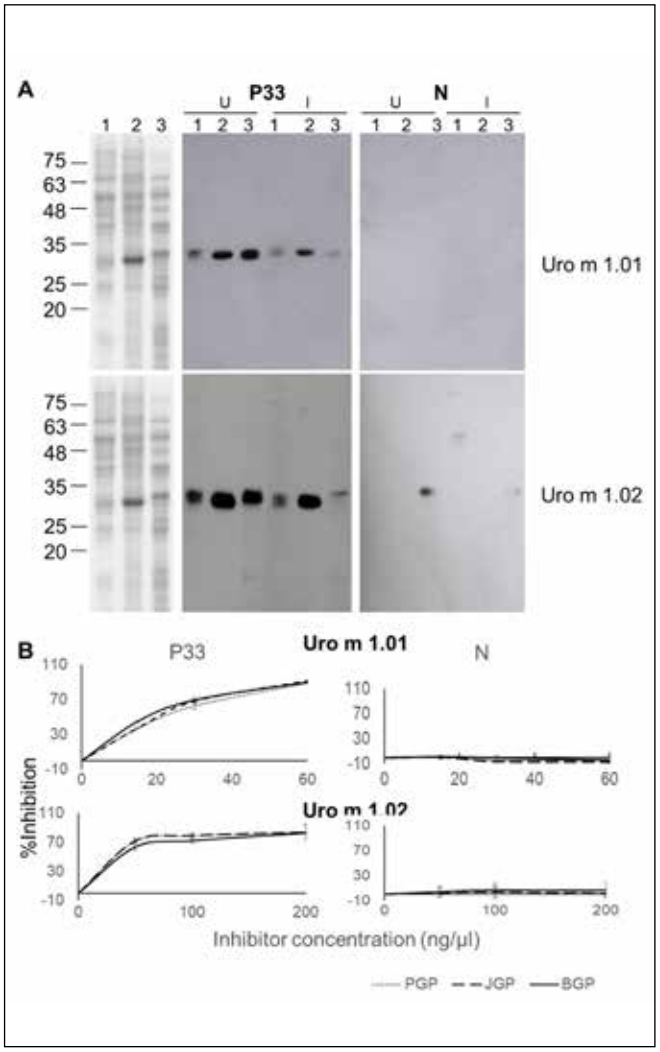


be achieved mostly due to the low concentrations of expressed recombinant proteins. These data confirmed that 1) the major IgE binding proteins in para grass crude extract were beta-expansins; 2) both isoforms of rUro m 1 could sufficiently inhibit the IgE binding to the native beta-expansin, suggesting significant cross reactivity; and 3) because the inhibition by each isoform was not complete, it is possible that other beta-expansins (or other 30 kDa proteins) were involved in the IgE binding.

Cross reactivity between rUro m 1 isoforms with Johnson and Bermuda GP proteins

Because group 1 GP allergens or beta-expansins are major allergenic proteins in all grass species, we would like to investigate the extent of IgE cross-reactivity among subtropical grasses using rUro m 1. The immunoblot and ELISA inhibitions were performed with crude extracts from pollen of Para, Johnson, and Bermuda grasses (PGP, JGP, and BGP). Serum number 33 was used to investigate the IgE-cross reactivity due to high specific IgE among these three grass species. Without inhibition, the IgE-binding signals of 30 kDa proteins were observed in all crude pollen protein extracts, but the signal was stronger for BGP and JGP than for PGP (**figure 5a**). When sera were pre-incubated with rUro m 1.01 and rUro m 1.02, sIgE binding was partially inhibited for JGP, and mostly removed for BGP and PGP. In ELISA inhibition assay, both Uro m 1.01 and Uro m 1.02 had strong inhibitory effects in dose-dependent manner for all GP proteins (**figure 5b**). Levels of inhibition by Uro m 1.01 and Uro m 1.02 were similar: IC_{50} calculated from log-concentration scale was of 24.3 ng/µl for Uro m 1.01, and extrapolated calculated from equation y = 18.712 ln(x) - 4.8524 was 18.8 ng/µl for Uro m 1.02. Therefore, these two beta-expansins might be the potent isoforms that play a major role in cross-reactivity among subtropical grass pollens.

Figure 5 *- Cross-inhibition of specific IgE binding to PGP, JGP, and BGP with rUro m 1. The immunoblot inhibition of IgE-binding was performed using sera pre-incubated with recombinant (I) in comparison with sera pre-incubated with buffer (U) (A). Percentage inhibitions of specific IgE binding to coated PGP, JGP, and BGP extracts were detected in ELISA analysis (B). The data were performed in duplicate and presented as mean ± SD. Lanes 1, 2, and 3 are PGP, JGP, and BGP extracts, respectively.*



Discussion

Urochloa mutica or para grass is a tropical/subtropical grass with extremely wide range of distribution. Its pollen extract had been shown to trigger positive skin prick test results in a considerably large number of AR patients in Thailand (16,17). Curiously, there had been no report of PGP allergy from other countries so far. It is unclear whether this is due to the relatively less prevalent distribution, pollen dispersion, or simply lack of information. More attention should be paid to the allergenicity of this species due to its invasive nature and the ability to adapt to new environment.

This study confirmed that the grass group 1 allergen (beta-expansin) was a major allergen of PGP. Furthermore, three different isoforms of the PGP allergen Uro m 1 were cloned and expressed as Uro m 1.01, Uro m 1.02, and Uro m 1.03 and were found to differ in their IgE reactivity. Although there have been reports of isoallergens found in other grass species such as *Brachypodium distachyon*, maize, Johnson grass, and rice (4,7,8,32), few other isoallergens from the same species had been tested for IgE reactivity in parallel. Similar to beta-expansin isoallergens in Bermuda grass (Cyn d 1) and Bahia grass (Pas n 1), Uro m 1 with

acidic to neutral pI (Uro m 1.01 and Uro m 1.02) had higher IgE reactivity than the basic isoform (Uro m 1.03) (6,33,34). Although Uro m 1.03 did not show potent IgE-binding function, this isoform could still be clinically relevant if sera from a larger or different population were tested. It is also possible that Uro m 1.03 was not expressed in a form that allowed IgE binding to be detected. In fact, Uro m 1.03 had 87% identity with Pas n 1.0101, which had been shown to be a major allergen in Bahia grass pollen (9). rPas n 1.01 which is a basic isoform shows frequency of 85% spIgE reactivity allergic rhinitis patient sera (9). Therefore, the basic isoforms are allergenic in some cases.

The three Uro m 1 isoforms obtained in this study were cloned based on PCR amplification of cDNA using specific primers designed from conserved regions of previously reported group 1 GP allergens, and were by no means exhaustive. In comparison with the most related species, maize, Uro m 1.01 and Uro m 1.02 could potentially be UmEXPB10 or UmEXPB11 and Uro m 1.03 could possibly be UmEXPB1. ZmEXPB1, ZmEXPB9, ZmEXPB10, and ZmEXPB11 are expressed in pollens and anthers and identified as group 1 grass pollen allergens in maize (8,15,35).

Based on the studies in species with complete genome information, including maize, rice, and *B. distachyon*, a number of beta-expansin genes are present within each genome (15,32,36). For example, in maize genomic sequence, three ZmEXPB10s were found on chromosome 3 and 9. Five ZmExpB11s were located in close proximity on chromosome 5, whereas ZmEXPB9 was located in a single cluster with three ZmEXPB1s on chromosome 9 (15). Thus, the three isoforms of Uro m 1 were also likely to be encoded by different loci in the genome and these proteins were possibly present together in the same pollen. Group 1 grass pollen allergens in maize (Zea m 1) were classified into two classes: class A (ZmEXPB10 and 11) and class B (ZmEXPB1 and 9), sharing about 60% sequence identity between classes (8,15). Likewise, our clustering analysis showed that Uro m 1 were divided into class A (Uro m 1.01 and 1.02) and class B (Uro m 1.03), along with isoforms of Sor h 1 and Pas n 1 (Panicoideae), and Ory s 1 (Ehrhartoideae). However, only class A isoforms had been reported for most allergenic grass species from Pooideae such as Phl p 1, Lol p 1, Poa p 1, Pha a 1, and Hol l 1; and Cyn d 1 from Chloridoideae, despite the high possibility that all Pooideae and Chloridoideae grass genomes also harbor class B beta-expansin genes. The absence of reported allergenic class B beta-expansins from Pooideae and Chloridoideae could suggest that only class A proteins have clinical relevance in these subfamilies. Until further information becomes available, it is unclear whether the IgE-reactive class B proteins are limited to grasses in Panicoideae subfamily.

Of the class A beta-expansins Uro m 1.01 and 1.02, the IgE reactivity was estimated to be high (all 6 of the 17 patients sensitized to PGP) among GP allergic patients. Because these two isoforms had highly similar sequences and pI values, the levels of IgE reactivity were also comparable. Both isoforms had the ability to significantly (up to 90-95%) inhibit IgE binding to beta-expansins in the crude extracts of PGP, BGP, and JGP. However, the actual contribution of each isoform to patient sensitization and elicitation of symptoms could not be discerned easily. Other factors such as gene expression level and protein modification could play important roles. Moreover, IgE reactivity could also differ in other populations. For example, Pas n 1 showed different IgE-reactivity levels in sera from patients of sub-tropical and temperate regions (37).

The ability of Uro m 1.01 and 1.02 to inhibit IgE binding of JGP and BGP extracts suggested that reactivity with Uro m 1 could be due to primary sensitization or co-sensitization with JGP and BGP, and cross-reactivity with PGP. Interestingly, the amino acid sequence of Uro m 1.01 is 100% identical to the sequence of Cyn d 1.0203. This result provided additional evidence that two allergens from different species may be more similar in sequence, and presumably in allergenicity, than two isoforms from the same species. Nonetheless, SPT using PGP and BGP extracts yielded different results. In our study, most patients who tested positive with PGP extract also tested positive with BGP (17/17) and JGP (15/17) extracts, but the reverse was not true (19). This could be due to the additional allergenic proteins present in the BGP and JGP or the different relative levels of allergenic proteins present in the GP crude extracts from these grass species.

In the era of precision medicine, it has been increasingly popular to identify the exact allergenic proteins or even the binding epitopes for each patient. Based on this study and others about grass group 1 allergens, it could be suggested that the class A and class B beta-expansins have different IgE reactivity and both classes should be represented when performing component-resolved diagnostics and allergen-specific immunotherapy.

Conflict of interest

The authors declare that they have no conflict of interest.

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