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ORIGINAL ARTICLE

Beta-Expansin of Bermuda, Johnson, and Para grass pollens, is a major cross-reactive allergen for Allergic Rhinitis patients in subtropical climate

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Abstract

Background: Subtropical grass pollens of Bermuda (BGP), Johnson (JGP), and Para or buffalo grass (PGP), are common causes of pollen allergies in warm climate area. Allergic rhinitis (AR) patients had positive skin prick test (SPT) to extract of these 3 grass pollens. However, no allergenic proteins of 3 grass pollens have never been studied.

Objective: To identify major allergens of BGP, JGP, and PGP in Thai grass pollen-allergic patients and to examine their sIgE cross-reactivity.

Methods: Serum of nine AR patients with positive SPT to at least 2 of 3 studied pollens were collected. Based on availability, only ImmunoCAP of BGP and JGP were available to determine a level of sIgE. Profiles of sIgE bound proteins from BGP, JGP, and PGP, were obtained by immunoblot. Major IgE bound protein was identified by liquid chromatography-tandem mass spectrophotometry (LC-MS/MS). Cross-reactivity of purified major allergen of the 3 grass pollens was determined by inhibition of sIgE in both ELISA and immunoblot.

Results: AR patients who have positive SPT to extract of BGP, JGP, and PGP, were 9, 8, and 6, respectively. Positive sIgE (> 0.35 kUA/L) to BGP and JGP were found in 9 and 8 patients, respectively. Eight profiles of IgE bound proteins of the 3 grass pollens showed 29-30 kDa pollen protein as major allergen and was identified as beta-expansin (ExpB). Moreover, purified ExpB of the 3 grass pollens cross-inhibited serum sIgE.

Conclusion: ~30 kDa ExpB of BGP, JGP, and PGP, is major cross-reactive allergen for AR Thai patients.

Keywords: Subtropical grass pollen allergens, pollen allergy, Allergic rhinitis, beta-expansin, allergic airway diseases, major airborne pollens

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Introduction

Allergens from grass pollens (GP) are one of major causes of allergic airway diseases, especially allergic rhinitis (AR).[1,2] Subtropical grasses in Chloridoideae subfamily (Bermuda grass, B, or *Cynodon dactylon*) and in Panicoideae subfamily (Johnson grass, J, or *Sorghum halepense*, and Para or Buffalo grass, P, or *Brachiaria mutica*, also known as *Urochloa mutica*), are commonly distributed in warm regions such as parts of Africa, Asia, Australia, United States of America (California and Florida), Central and South America.[2] BGP, JGP, and PGP are reported as a source of allergenic proteins triggering allergic inflammation in upper airway. To date, 7 allergens of BGP and 2 allergens of JGP have been reported.[2] However, to date, allergens of PGP have not yet been identified. In Thailand, pollens of grasses are one of major airborne pollens found in a high count throughout a year and are highest count in May

and September for grass pollen ≤ 40 μm and in September for grass pollen >40 μm.[3] It has been reported that, based on skin prick test (SPT), 52.3% and 49.4% of 736 Thai patients attending the ENT Allergy Clinic at Siriraj Hospital, had reaction to protein extract of BGP and PGP, respectively.[1] Moreover, It was recently reported that, based on SPT results, 45.6% of 68 Thai volunteers with respiratory allergy had reaction to JGP extract.[4] These findings indicated that allergens of BGP, JGP, and PGP, are one of major causes for respiratory allergy in sensitized Thai patients. Despite these results, however, pollen allergens from these 3 pollens sensitized by Thai patients have not yet been identified. Therefore, this study aimed to identify major allergenic proteins of BGP, JGP, and PGP, sensitized by Thai pollen-allergic patients as well as examine their cross-reactivity.

Methods

Patients

The study was approved by the Institutional Review Board, Siriraj Hospital (SiEc 100/2012). Forty patients with history of AR were recruited. All patients signed an informed consent before SPT. Thirty-one of them were excluded due to mono -sensitization to grass allergen. Nine patients, aged 9-47 years old (mean 26.3 ± 4 years), were included in this study based on positive SPT (mean wheal diameter, MWD ≥ 3 mm) to protein extract from at least 2 of 3 BGP, JGP, and PGP. The SPT extracts were prepared as described previously.[4] Determination of level of serum sIgE against extract of BGP and JGP was carried out by ImmunoCAP (Phadia, Uppsala, Sweden). However, there is no available test for PGP.

Pollen collection

Grass flowers were collected from various sites in Thailand. Flowers were dissected under a stereo-microscope and identified to species using identification key. Flowers were left for natural shedding for 1 day. Pollen grains were incubated for 24 h in a chamber containing silica gel before purified by passing through 120, 150, and 230 mesh-sieve. Only the pollen with 95% purity observed under the microscope were stored at -80°C until used.

Pollen crude extract preparation

Pollens were ground in liquid nitrogen and extracted in phosphate buffer saline (PBS) buffer containing 1 mM Phenylmethylsulfonyl fluoride (PMSF) with continuous stirring at 4°C for 1 h. The extract was centrifuged at 13,000 × g for 15 min and supernatant was collected before filtered through 0.45 micron filter. Protein concentration was determined using Bradford's assay. The efficacy and safety of this extract has been previously reported.[4]

Electro-elution of 29-30 kDa IgE bound pollen protein

Electro-elution was carried out using electro-eluter (Bio-Rad, USA). The preparative gels were carried in parallel with gels for immunoblot to ensure that correct protein bands were excised. The protocol was followed the instruction from the manufacturer. Twenty μg of pollen protein extract was loaded per well and resolved in 12% SDS-PAGE gel. This preparative gel was prepared with the same percentage and running condition as the gels prepared for immunoblot. Coomassie G-250 stained protein band matched IgE bound protein band of immunoblot was excised and placed in glass tube connected with pre-soaked membrane cap (3.5 kDa MWCO) where eluted protein was retained. The assembled glass tube was filled with elution buffer (25 mM Tris-HCl pH 8.2, 192 mM glycine and 0.1% (w/v) SDS) and placed into a tube holder. The holder was placed in buffer tank of Mini Transblot Electrophoresis Transfer Cell (Bio-Rad, USA) and protein was eluted from gel at a constant current of 8 mA/glass tube for 3-4 h. Eluted protein retained in the membrane cap was transferred and stored at -20°C. Analysis by SDS-PAGE gel and immunoblot were done to confirm its IgE binding activity before used in all IgE inhibition tests.

Identification of Major IgE bound protein by Liquid Chromatography-Tandem Mass Spectrophotometry (LC-MS/MS)

The following protocol was done per gel piece of one protein band. An excised gel piece was submerged in buffer A [50% Acetonitrile (ACN), 50 mM NH_4HCO_3] until colorless. The buffer A was replaced with 10 mM dithiothreitol (DTT) and incubated for 15 min at 60°C before was added with buffer B (55 mM iodoacetamide, 50 mM NH_4HCO_3) and incubated for 30 min at room temperatue in the dark. The 100% ACN was replaced to dry gel before adding trypsin solution [0.1mg/mL trypsin (Sigma-Aldrich, USA), 50 mM Ammonium bicarbonate] and incubated at 37°C overnight. The reaction was mixed with ACN at 1:1 (v/v) ratio and incubated for 20 min. All solution was transferred to a new tube and dried in centrifugal concentrator at 45°C. Resolved pellet in 0.1% formic acid was injected into an Ultimate 3000 nano-LC system (Dionex; Surrey, U.K.) coupled with Micro-ToF Q II mass spectrometer (Bruker; Bremen, Germany). The mass spectra data were acquired using Hystar software (Bruker Daltonics, Germany) and were converted by Compass Data-Analysis software (Bruker Daltonics, Germany). The converted files were analyzed with Mascot server (version 2.3.0, Matrix Science, USA) to search matched sequence in NCBI database with 95% confidence.

Direct IgE binding and Inhibition of IgE ELISA

Pollen extracts were coated at 500 ng/well of a 96-well microtiter plate and incubated at 4°C overnight. The coated plate was washed and incubated with 1/4-1/20 diluted serum of SPT positive patients and control for 2 h and washed before incubated with HRP-labeled mouse IgG anti-human IgE for 1 h. HRP substrate TMB (3,3′5,5-tetramethylbenzidine) was added and blue color reaction product was read at a wavelength of 650 nm.

For inhibition ELISA, 500 ng of eluted 30 kDa beta -expansin (ExpB) of BGP extract was coated per well. Pre -determined diluted sera of patients and control were incubated with 0.5, 1, 10, and 20 μg/ml of eluted ExpB (29-30 kDa protein) of BGP (CD29), JGP (SH29), and PGP (BM30), for overnight at 4°C before was centrifuged at 17,210 × g for 10 min at room temperature (RT). The supernatant was added to coated wells and followed the mentioned direct ELISA protocol.

Direct IgE Immunoblot and IgE inhibition immunoblot

Sera of patients and control were diluted at 1/10-1/50 in PBS containing 3% skimmed milk (buffer A) and used in the following tests.

For direct IgE immunoblot, 2 µg of pollen protein extract were resolved per well of 12% SDS-PAGE gel at constant current. Separated proteins in the gel were electro-transferred onto nitrocellulose membrane before incubated in buffer A for 1 h at RT. The membrane was washed with PBS containing 0.2% v/v tween-20 (buffer B) before incubated overnight at 4°C with diluted sera of patients and control in buffer A. The membrane was washed with buffer B and was incubated with 1:10,000 diluted horse-radish peroxidase (HRP) conjugated mouse IgG anti-human IgE antibody (KPL, MD, USA) in buffer A for 1 h. After washing, the membrane was incubated with HRP substrate (Millipore, MA, USA) and emitted signal was captured by x-ray film.

For IgE inhibition immunoblot, 1/50 diluted sera of patients and control were incubated with 20 µg of eluted ExpB for overnight at 4°C before was centrifuged at 17,210 × g for 10 min at RT. The supernatant was incubated with nitrocellulose membrane on which had transferred CD29, SH29, and BM30, for 2 h at RT and the remained steps were the same as those of direct immunoblot.

Results

Nine patients with AR were included in this study based on positive SPT to at least 2 of 3 extracts (**Table 1**). The mean age of patients was 26.3 ± 12.3 years. The severity of allergic rhinitis was not different between patients who had low or high specific IgE. Thus, all included patients were classified as mild persistent allergic rhinitis. All patients had positive SPT to BGP extract while 8 of 9 had positive SPT to JGP extract (**Table 1**). However, only 6 of 9 patients had positive to PGP pollen extract. The MWD of SPT to extract of BGP, JGP, and PGP, were 5.8 mm, 3.8 mm, and 3 mm, respectively. The results of ImmunoCAP showed all patients had sIgE > 0.35 kUA/L to BGP extract while 8 patients had sIgE > 0.35 kUA/L to JPG extract (**Table 1**).

To further determine profiles of IgE bound pollen proteins extracted from 3 grasses of all included patients, the results showed that 8 profiles had IgE bound protein mobilized between 25 and 37 kDa markers (**Figure 1**). One profile (Pt33) could not be determined due to a lack of serum. Two profiles

Table 1. Demographic of recruited Allergic Rhinitis individuals of this study

Pt. ID	sex	Age	SPT (mean wheal diameter, mm)			sIgE (kUA/L)	
			Bermuda	Johnson	Para	Bermuda	Johnson
9	F	46	9	5	3	30.3	34.7
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
39	M	25	4	3.5	2	1.23	0.51
40	F	41	7.5	3.5	4.5	0.85	0.55
43	F	33	6.5	4	2.5	0.73	0.04
47	M	13	3.5	2	3	11.2	11.4

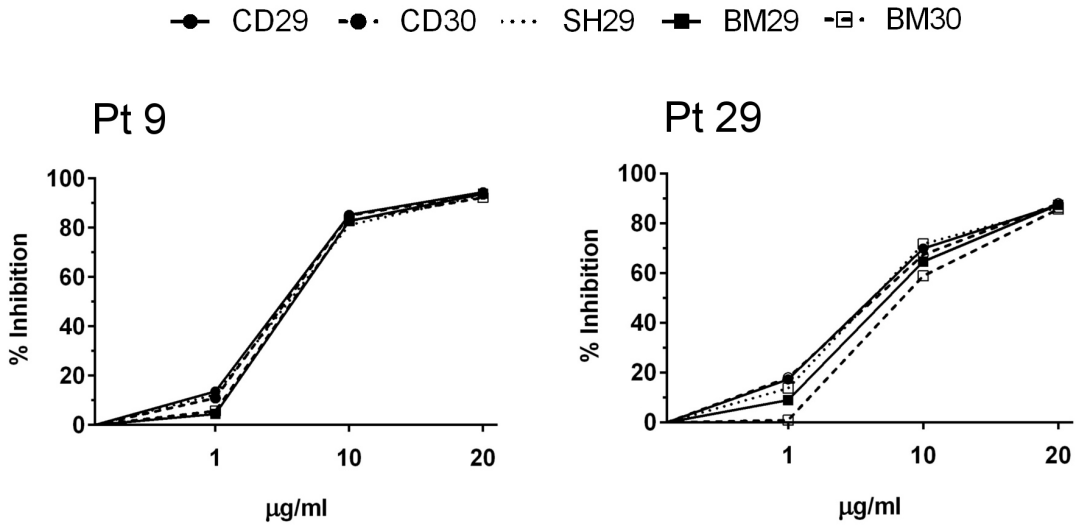

Figure 1. Profiles of IgE bound pollen proteins extracted from BGP, JGP, and PGP.
Note: total protein extract of grass pollens: 1 = BGP, 2 = JGP, and 3 = PGP.

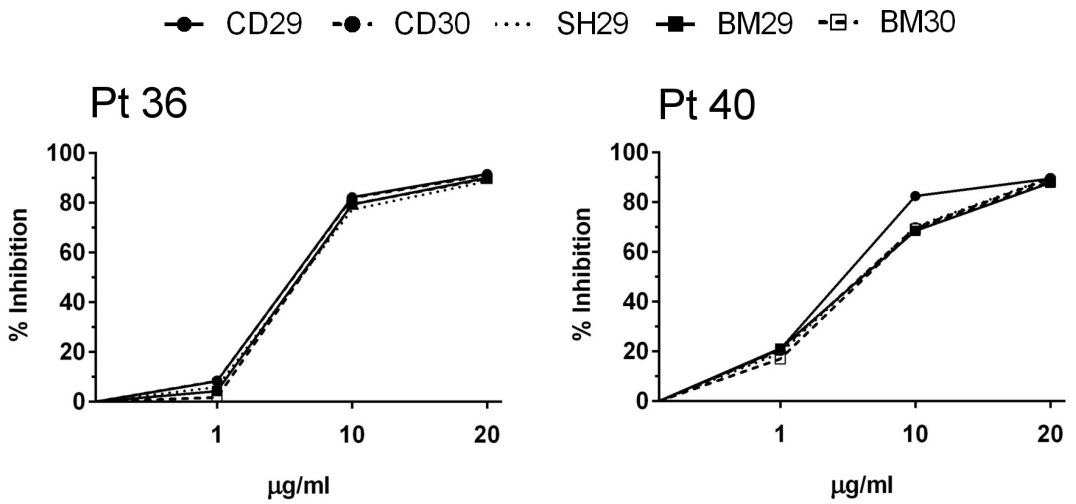

Figure 1. (Continued) Profiles of IgE bound pollen proteins extracted from BGP, JGP, and PGP.
Note: total protein extract of grass pollens: 1 = BGP, 2 = JGP, and 3 = PGP.

(Pt29 & Pt39) show IgE bound multiple proteins of 3 grass mobilized 37-50 kDa markers, while other five profiles show IgE bound multiple proteins of 1 grass mobilized 37-50 kDa markers (**Figure 1**). Moreover, three profiles (Pt17, Pt39 & Pt47) showed IgE bound multiple proteins of 3 grass mobilized 10-20 kDa markers (**Figure 1**). Based on these IgE bound protein profiles, protein mobilized between 25-37 kDa appears to be a major allergen among the 3 grass and was subjected for LC-MS to identify its identity.

The results of LC-MS/MS showed that 2 excised proteins (CD29, 30) of BGP extract matched 5-7 peptides of ExpB or Cyn d 1 (**Table 2**). Excised protein of JPG (SH29) and PGP (BM29, 30) extract matched 2-5 peptides of expansin B1 of corn (Zea mays) (**Table 2**). Moreover, the sequence of BM30 also matched 4 peptides of expansin B9 of japanese rice (*Oryza sativa subsp. japonica*) (**Table 2**). These results suggested that a protein of ExpB family is the major allergen of BGP, JGP, and PGP, sensitized by Thai AR patients. To confirm

Table 2. LC-MS identified IgE bound protein of 3 grass pollens

ID	protein	mass	Protein score	peptide sequence
CD29	Cyn d 1	26872	153	SSWGAIWR ATFYGSNPR AGELTLQFR KPLKGPFSIR KAGELTLQFR CKEPVECSGEPVLVK
CD30	Cyn d 1	26872	140	SSWGAIWR ATFYGSNPR AGELTLQFR DSDEFIPMK KPLKGPFSIR
SH29	Expansin-B1 Zea mays	29066	137	GCGSCYEVR AFGSLAKPGLNDK AFGSLAKPGLNDKIR NVNLPPYSGMTACGNVPIFK NVNLPPYSGMTACGNVPIFKDGK
BM29	Expansin-B1 Zea mays	29066	65	LSWGAIWR ALKGPFSIR AFGSLAKPGLNDK AFGSLAKPGLNDKIR
BM30	Expansin-B1 Zea mays	29066	42	LSWGAIWR AFGSLAKPGLNDKIR
	Expansin-B9 Oryza sativa subsp. japonica	28966	34	LSWGAIWR AGIIDMQFR KAGIIDMQFR AGIIDMQFRR

Note: Matching of amino acid sequence using Mascot server (version 2.3.0, Matrix Science, USA) with 95% confidence.

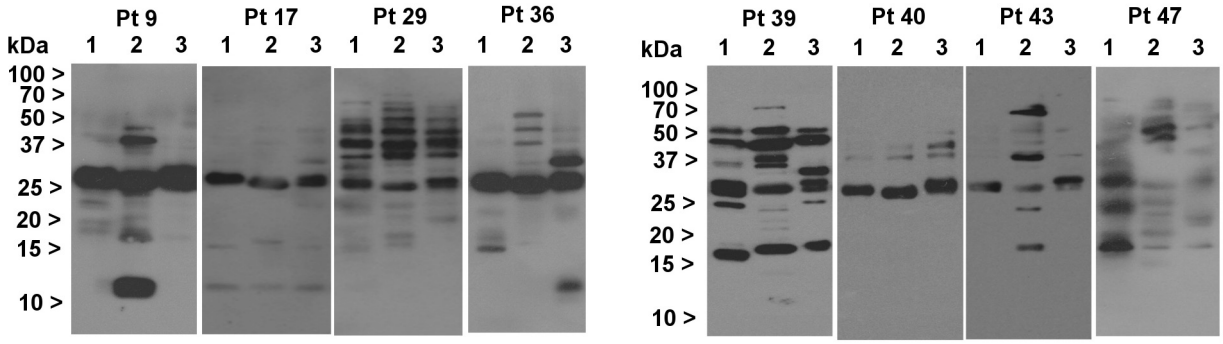

Figure 2. Cross-inhibition of specific IgE binding to coated CD29 with CD30, SH29, BM29, and BM30.
Note: serum of 4 individuals: Pt9, Pt29, Pt36, and Pt40; specific IgE binding was inhibited at an average IC_{50} of 6 µg/ml.

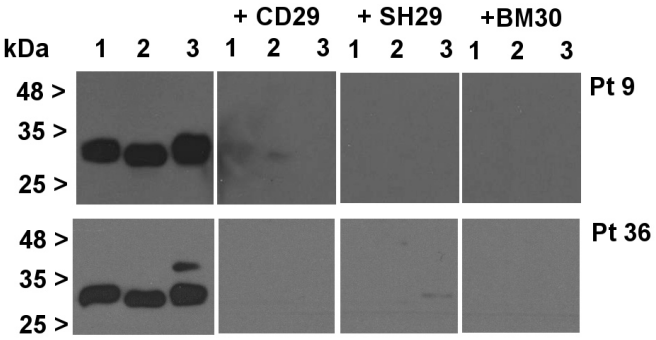

Figure 3. Inhibition of specific IgE to ExpB of 3 grass pollens with eluted ExpB.
Note: serum specific IgE of Pt9 and Pt36; 1 = eluted CD30; 2 = eluted SH29; 3 = eluted BM30; +CD29, +SH29, and +BM30 = incubated serum with eluted CD29, SH29, and BM30, respectively.

IgE cross-reactivity of ExpB of the 3 grass pollens, inhibition of sIgE binding to CD29 by CD30, SH29, BM29, and BM30, were determined. The results of inhibition ELISA showed all 5 proteins were able to cross-inhibit sIgE binding to coated CD29 at an average IC_{50} of 6 µg/ml (**Figure 2**). Moreover, the results of inhibition immunoblot also showed complete inhibition of IgE binding to CD29, SH29, and BM30 by the same 3 proteins (**Figure 3**).

Discussion

This is the first study to report that ExpB of BGP, JGP, and PGP, is major IgE cross-reactive allergen for grass pollen allergic Thai AR patients. It has been reported that ExpB or Cyn d 1 of BGP appears to be major grass pollen allergen in many subtropical areas.[5] The Cyn d 1 cross-inhibited sIgE binding to ExpB of subtropical Bahia grass (Pas n 1) with strong affinity while to that of temperate Ryegrass (Lol p 1) with moderate affinity.[5,6] The difference in IgE affinity to group 1 allergen of subtropical grass and temperate grass may be caused by different subfamily. In subtropical grasses, Bermuda is in Chloridoideae subfamily while both Johnson and Para are in Panicoideae subfamily. Both subfamilies are from the same lineage (PACMAD clade) in phylogenic tree and ExpB of the 2 subfamilies shared 65% identity of amino acid sequence. Based on the LC-MS results, identical amino acid sequences between ExpB of BGP and PGP are WGAIWR and LKGPFSIR, located in the C-terminal domain of Cyn d 1.[8,9] Moreover, the peptide, WGAIWR (aa194-199) was showed to be one of major IgG4 binding sites on Cyn d 1.[9] Despite a moderate sequence identity, ExpB of these 3 grass pollens could cross-inhibit sIgE to ExpB with strong affinity (IC_{50} of 6 µg/ml). It has been reported that IgE cross-reactivity between group 1 of subtropical grass and that of temperate grass could also trigger allergic reaction in patients from either subtropical or temperate regions.[10,11] Based on these results, thus, ExpB of one grass species, such as Cyn d 1, could be a good allergen used in diagnosis and immunotherapy of AR patients who sensitize to subtropical grass pollens from grass of PACMAD clade. Moreover, several agricultural crops, such as maize, sorghum, sugar cane, and pearl millet, are belonged to the same Panicoideae subfamily as Johnson and Para grass. Thus, investigation of IgE cross-reactivity of ExpB in pollens from mentioned agricultural crops would be essential because they are economically important crops for countries in warm regions and many field workers may likely suffer pollen-caused AR.[2]

Results of SPT and ImmunoCAP appear to correlate well as 7 of 9 patients had a value of sIgE against BGP and JGP grass pollen ≥ 0.35 kUA/L while had ≥ 3 mm MWD in SPT. However, the other 2 patients did not have all positive results from both testes, for instance, Pt8 had SPT positive to both BGP and JGP but sIgE to only BGP or, for Pt9, SPT positive to BGP but sIgE to both BGP and JGP (**Table 1**). Although values of sIgE and SPT of 7 patients indicated they are allergic to proteins of BGP and JGP, however, values of these 2 tests may not predict results of one another. For instance, Pt4 had SPT value of 8.5 mm to BGP and 4.5 mm to JGP,

while sIgE values were > 100 and 85.3 kUA/L to BGP and JGP, respectively. In contrast, Pt7 had SPT value of 7.5 mm to BGP and 3.5 mm to JGP, while sIgE values were 0.85 and 0.55 kUA/L to BGP and JGP, respectively. Based on these results, it appears that SPT could be sufficient test to diagnose pollen allergy as also reported by others.[1,4-7,10,11] Pollen extract in this report was prepared from 95% purified pollens from identified grass species.[4] Therefore, results from SPT and IgE reactivity assays, such as ELISA, would be a result from allergens interacting sIgE.[1,4-7,10,11]

In conclusion, ExpB of Bermuda, Johnson, and Para grass pollens is the major cross-reactive allergen for Thai patients with AR and could be one of essential components for diagnosis of pollen allergy.

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