APJAI
Asian Pacific Journal of
Allergy and Immunology

ORIGINAL ARTICLE

Manila grass (*Zoysia matrella*) Zoy m 1 allergen may contribute to allergic sensitization in tropical/subtropical regions due to extensive cross-reactivity with other group-1 grass pollen allergens

Koravit Somkid,[1,7] Sirirat Aud-in,[2,3] Bannapuch Pinkaew,[4] Pongsakorn Tantilipikorn,[4] Surapon Piboonpocanun,[5] Wisuwat Songnuan[2,6,7]

Abstract

Background: Pollen of grasses in Chloridoideae and Panicoideae subfamilies is a major source of grass group-1 allergens in tropical/subtropical areas. Previously, most studies of subtropical grass pollen allergens have focused on *Cynodon dactylon* (Bermuda grass-Chloridoideae) and *Sorghum halepense* (Johnson grass-Panicoideae). However, little information is available about allergenicity of pollen from *Zoysia matrella* (Manila grass or Zoysia grass-Chloridoideae), which is among the most popular turfgrasses in tropical/subtropical areas.

Objective: This study aimed to investigate the IgE reactivity and cross-reactivity of grass group-1 allergen from *Z. matrella*. In addition, the clinical relevance of *Z. matrella* in comparison with other species was assessed.

Methods: IgE reactivity and cross-reactivity between recombinant proteins of group-1 allergen from *Z. matrella* (Zoy m 1) and *C. dactylon* (Cyn d 1) were determined by ELISA and immunoblot assays. Clinical relevance of *Z. matrella* pollen in Thai atopic patients was assessed using its pollen crude extract for skin-prick test, in comparison with extracts from four other pollen species.

Results: The Zoy m 1 had high IgE binding and could interfere with binding to *C. dactylon* crude extract. In addition, *Z. matrella* pollen extract elicited positive skin-prick test results comparable to previously reported allergenic species. Group-1 grass pollen allergen was confirmed to be a major allergen from *Z. matrella* among Thai atopic patients and was officially designated Zoy m 1.0101.

Conclusion: Zoy m 1 allergen is a major allergen from *Z. matrella* that cross-reacts with other group-1 grass pollen allergens in the tropical/subtropical region.

Key words: Zoysia grass; Chloridoideae; subtropical grass; allergic rhinitis; cross-reactivity; Zoy m 1

From:
[1] Toxicology graduate programme, Faculty of Science, Mahidol University, Bangkok, Thailand
[2] Department of Plant Science, Faculty of Science, Mahidol University, Bangkok, Thailand
[3] Department of Pharmaceutical Botany, Faculty of Pharmacy, Bangkok, Thailand
[4] Rhinology & Allergy Division, Department of Otorhinolaryngology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
[5] Institute of Molecular Biosciences, Mahidol University, Salaya Campus, Nakhon Pathom, Thailand
[6] Systems Biology of Diseases Research Unit, Faculty of Science, Mahidol University, Bangkok, Thailand
[7] Center of Excellence on Environmental Health and Toxicology, Bangkok, Thailand (EHT)

Corresponding author:
Wisuwat Songnuan
Department of Plant Science, Faculty of Science, Mahidol University, Bangkok 10400, Thailand
Email: wisuwat.son@mahidol.edu

Introduction

Grass pollen is one of the most significant aeroallergen sources causing allergic respiratory diseases such as asthma and allergic rhinitis worldwide. Most pollen allergy studies have focused on grass species in the Pooideae subfamily distributed mainly in the temperate climate zone. These studies pinpointed key allergenic species, including Timothy grass,

rye grass, orchard grass, canary grass, and velvet grass. Furthermore, the major allergens and serum IgE cross-reactivity of these grasses have been extensively characterized.[1,2,3,4] Two other grass species considered to be major sources of allergenic pollen in the temperate regions are Bermuda grass (*Cynodon dactylon*) from Chloridoideae subfamily and Johnson grass (*Sorghum halepense*) from Panicoideae subfamily.

The grass group-1 allergens or beta-expansins are major allergens with the highest potency and prevalence among Panicoideae and Chloridoideae grasses, and the similarity of these allergens could indicate the level of cross-reactivity.[5,6,7] Of all group-1 allergens from these two grass subfamilies, Cyn d 1 from *C. dactylon* and Sor h 1 from *S. halepense* are the most well-characterized allergens.

In comparison, knowledge about grass pollen allergen sources in tropical/subtropical zones is scarce, especially in Asia Pacific region, where genetic diversity of grasses is substantially higher. Notably, Pooideae grass species are rarely present in this region. In Thailand, grass pollen can be found throughout the year, with high peaks in May and September.[8] The identity of major species contributing to the airborne grass pollen and grass pollen sensitization, however, cannot be discerned from similar-looking pollen captured in the survey. To overcome this hurdle, pollen from prevalent candidate species must be individually tested for IgE reactivity and allergenicity. Based on skin prick test results from ENT Allergy Clinic at Siriraj Hospital, Bangkok, *C. dactylon* and *S. halepense* had been identified as important allergen sources, along with *Urochloa mutica* (Para grass), another member of the Panicoideae subfamily.[9]

Manila grass [*Zoysia matrella* (L.) Merr.], another member of the Chloridoideae subfamily, is found in high abundance in several countries in Africa, North America, Central America and Caribbean, South America, Asia and Oceania, where it has become one of the most popular choices for turfgrass.[10] Recently, we sequenced the cDNA of group-1 grass pollen allergen from *Z. matrella* and showed that it was almost identical to previously characterized allergens.[11] However, no information about *Z. matrella* pollen allergenicity has been reported. This study aimed to assess the IgE reactivity and cross-reactivity of group-1 allergen from *Z. matrella*. Furthermore, a skin prick test using crude extract of the *Z. matrella* grass pollen, alongside other grass pollen extracts, was performed to determine its clinical relevance.

Materials and Methods

Patient selection

For the immunoreactivity and cross-reactivity study, patients > 18 years old with allergic rhinitis and/or asthma symptoms were recruited at the ENT clinic, Siriraj Hospital, Bangkok, with written informed consent. Exclusion criteria include chronic diseases, skin lesions around testing areas, history of anaphylaxis, and pregnancy. Patients were subjected to skin prick testing (SPT) with crude extracts from *Cynodon dactylon* (CD), *Urochloa mutica* (UM), *Amaranthus hybridus* (AH), and *Cyperus mitis* (CM). Normal control samples were obtained from non-atopic donors with negative SPT results with *C. dactylon* pollen extract. Sera from seven patients with positive SPT results with *C. dactylon* pollen extract (wheal diameter ≥ 3 mm) were randomly selected for this study. Serum samples were collected and kept at -80°C until further used. The protocol of this study was approved by an ethics committee of the Institutional Review Board, Siriraj Hospital (Si 171/2017) before recruitment of the subjects. Demographics of the recruited patients whose sera were used for the immunoreactivity and cross-reactivity study is shown in **Table 1**. tapatient sensitization to different grass pollen extracts, > 18 years old patients with allergic rhinitis and/or asthma were subjected to skin prick test using grass pollen extracts from five species: *Z. matrella* (ZM), *C. dactylon* (CD), *S. halepense* (SH), *U. mutica* (UM), and *B. pertusa* (BP). Exclusion criteria were the same as those previously described for the immunoreactivity and cross-reactivity study. The study protocol was approved by an ethics committee of the Institutional Review Board, Siriraj Hospital (Si 476/2016). Demographics of the recruited patients are shown in **Table 2**.

Table 1. Demographics and SPT results of *C. dactylon* pollen allergic patients whose sera were chosen for immunoblotting and ELISA inhibition in this study.

Patient number	Age	Sex	SPT (mean wheal diameter (mm))			
			Cynodon dactylon	*Urochloa mutica*	*Amaranthus hybridus*	*Cyperus mitis*
2	23	M	3	4.5	(0)*	3.5
4	21	M	4	(0)*	(0)*	5
10	26	F	3	3	(0)*	(0)*
11	23	F	3	(0)*	(2.5)*	3
12	38	F	4	6.5	(2.5)*	4
13	47	F	5.5	3.5	(0)*	(0)*
16	32	F	4.5	5.5	3	(0)*

*Numbers in parentheses indicate negative results with wheal diameter < 3 mm

Table 2. Demographic profiles of patient cohort tested for sensitization to grass pollen.

Characteristics	Total	Positive SPT to pollen	Positive SPT to grass pollen
No. of Patients	168	94	70
Age (years)			
- Mean	38.39	36.27	35.56
- Range	15-67	15-67	15-66
Sex			
- Male	61	41	32
- Female	107	53	38
- Ratio M:F	1:1.75	1:1.24	1.19

*Numbers in parentheses indicate negative results with wheal diameter < 3 mm

Pollen collection

Inflorescences of all five species of grass were collected from various natural sites in Thailand. Grass species were identified by taxonomists and voucher specimens were kept at the Department of Plant Science, Faculty of Science, Mahidol University. Pollen released from inflorescences was gathered and incubated in silica gel for 24 hours. Gathered pollen was purified from contaminating plant parts (until > 99% purity) using a series of sieves. The purified pollen was stored at -80°C until used.

RNA extraction and cDNA synthesis

Total RNA was extracted from 100 mg of *C. dactylon*, *Z. matrella*, and *S. halepense* grass pollen. Pollen was ground in a chilled mortar with TriZol® reagent (Thermo Fisher Scientific, USA) and further steps were performed according to the manufacturer's protocol. The concentration and quality of RNA was measured by Nanodrop™ spectrophotometer (Thermo Fisher Scientific, USA). RNA was kept at -80°C until used. cDNA was synthesized from an RNA template using iScript™ cDNA kit with oligo [dT] primer.

Recombinant protein cloning and expression

The group-1 allergens from *Z. matrella* and *C. dactylon* were amplified from previously constructed and sequence-verified pGem®-T vector[11] using specific primers shown in **Table 3**. Amplicons were cloned into a pET-28a vector using EcoRI and XhoI restriction sites and transferred into Rosetta™ (DE3) competent cells. Positive clones were sequenced to confirm that the selected plasmids contained no error. Protein expression was induced using IPTG at 0.4 mM for 6 hours at 37°C. Recombinant His-tagged proteins were purified using a Ni-NTA column (Thermo Fisher Scientific, USA). The purified proteins were analyzed and quantified by SDS-PAGE and Bradford's assay. A previously reported recombinant protein rD7,[12] a major allergen from *Aedes aegypti*, was used as a negative control.

Preparation of grass pollen crude extracts

For the immunoreactivity and cross-reactivity study, total protein was extracted from 100 mg of macerated pollen of *C. dactylon*, *S. halepense*, and *Z. matrella*, in PBS containing Phenylmethylsulfonyl fluoride (PMSF) at final concentration of 1 mM. The mixture was centrifuged at 12,000 rpm for 15 minutes to remove unwanted materials. The extracted protein profile was analyzed using SDS-PAGE. Protein concentration was measured using Bradford's assay. The extract was stored at -20°C until used for further analysis. For skin prick testing, crude pollen extracts at 10,000 PNU/mL were prepared in-house by the Department of Pharmacy, Faculty of Medicine, Siriraj Hospital as previously described.[13]

ELISA and Inhibition assay

The extracted protein of *C. dactylon* pollen was used to coat a 96-well plate at a concentration of 1 µg/well. The coated plate was incubated overnight at 4°C, washed and blocked with skim milk. Sera from allergic patients and normal control donors were added into the coated wells at ¼ dilution with a 2-hour incubation and then washed off. HRP-labeled mouse anti-human IgE antibody was added and incubated for one hour followed by another wash step before the addition of TMB substrate. The resulting color was measured at 650 nm. The inhibition assay was performed in a similar manner except that patient serum was pre-incubated with recombinant protein overnight and centrifuged before use.

Direct IgE immunoblot and IgE inhibition immunoblot

Briefly, 10 µg/well of *C. dactylon* pollen extract and 5 µg/well of Cyn d 1 and Zoy m 1 recombinant proteins were separated on SDS-PAGE gel and electro-transferred to nitrocellulose membranes using Trans-Blot® Turbo™ Transfer System (Bio-Rad, USA). The membranes were incubated with a blocking buffer for 1 hour, washed for 5 times with a washing buffer, and then incubated with individual serum diluted in the blocking buffer (1:50 to 1:150). Membranes were washed

Table 3. Gene specific primers for recombinant protein expression.

Primer	Sequence (5′-3′)	Tm (°C)	Product
EX004	F: GCCAAGCTTGAATTCATGGCCTCCTCCTCAGCG	67.7	Zoy m 1
EX005	R: GCCAAGCTTCTCGAGTCAGTACTGAAGGTTGGAGGT	66.6	
EX006	F: GCCAAGCTTGAATTCGAACAGGCTATGCTCGCG	66.6	Cyn d 1
EX007	R: GCCAAGCTTCTCGAGTCAGAACTGGATCTTGGACTTGTAGAC	66.4	

Abbreviations: F: forward, R: reverse.

5 times before the addition of HRP-labeled mouse IgG anti-human IgE antibodies (KLP, USA) diluted in blocking buffer (1:5,000). The bound IgE was detected with Immobilon™ Western Chemiluminescent HRP substrate (Millipore, Germany) and visualized with x-ray film (Amersham Bioscience, USA). Inhibition immunoblot assay was performed in a similar manner, except that sera were pre-incubated overnight with 200 μg/ml of Cyn d 1 or 500 μg/ml of Zoy m 1. After incubation, the mixture was centrifuged at 15,000 × g for 10 min and the supernatant was used in the immunoblot assay as previously described. The recombinant protein of mosquito *Aedes aegypti* (rD7) that was expressed and purified in a system comparable to that of Cyn d 1 and Zoy m 1 proteins was used as a negative control.

Results

Protein profile and IgE reactivity of Z. matrella pollen crude extract

SDS-PAGE analysis was used to assess the overall pollen protein profile of *Z. matrella* in comparison with *C. dactylon*, and *S. halepense* (**Figure 1A**). The crude protein extracts from all three species contain a distinct band at approximately 30 kDa, the expected size of group-1 allergen (beta-expansin), with a notably higher band intensity in the *S. halepense* than *C. dactylon* and *Z. matrella* extracts (**Figure 1A**). IgE reactivity of *Z. matrella* pollen extract was shown in comparison with that of *C. dactylon* in **Figure 1B**. Due to the unavailability of patient serum with sensitivity to *Z. matrella* pollen, the sera from AR patients with sensitivity to *C. dactylon* were used in this immunoblot. The demographic information about these patients is shown in **Table 1**. For the immunoblot of *C. dactylon* pollen extract, two prominent IgE-interacting bands at approximately 30 and 40 kDa could be seen with all positive sera, with the additional 20-22 kDa band in 2/7 positive sera. The observed patterns were less uniform for the immunoblot of *Z. matrella* pollen extract, in which multiple faint bands were observed that varied among the positive sera. Notable band sizes were 20-26, 26, 30, 32, 50-55 kDa. At the expected sizes of group-1 allergen, a single IgE interacting band with a faint shadow band was observed at approximately 30 kDa for *C. dactylon*, whereas two clear bands were detected at 30 and 32 kDa for *Z. matrella*. The group-1 allergen from *Z. matrella* was officially designated Zoy m 1.0101, and will henceforth be referred to as Zoy m 1.

IgE reactivity and cross-inhibition capability of recombinant Zoy m 1 protein

The beta-expansin of *C. dactylon* (Cyn d 1) has been extensively shown to have high IgE reactivity. It was questioned whether Zoy m 1 has similar IgE reactivity to that of Cyn d 1 due to a highly similar amino acid sequence with only a few substitutions located within the IgE and IgE/IgG4 binding epitopes (**Supplemental figure 1**).[14,15,16,17] To answer this question, recombinant Cyn d 1 and Zoy m 1 were produced and compared in immunoblotting assay using sera from atopic patients allergic to *C. dactylon* pollen. Induction of Cyn d 1 and Zoy m 1 in the *E. coli* system and the following purification steps are shown in **Supplemental figure 2A and 2B**, respectively. The IMAC purification yielded recombinant proteins with 84.52% purity for Cyn d 1 and 88.93% for Zoy m 1.

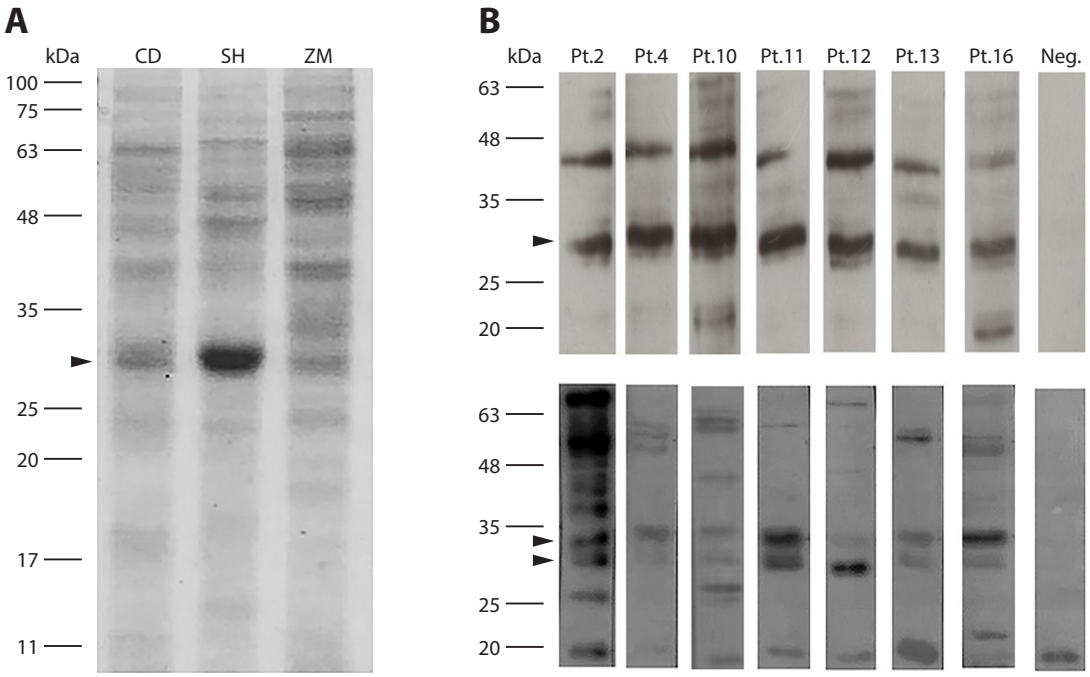


Figure 1. The whole pollen protein extracts of three grass pollen were visualized by SDS-PAGE analysis, when lanes CD, SH, and ZM are *C. dactylon*, *S. halepense*, and *Z. matrella* extracts, respectively (A). IgE-immunoblot profiles of CD (upper) and ZM (below) grass pollen crude extracts using sera from seven grass pollen atopic patients in *C. dactylon* (B). Note: Sera from 7 patients (Pt.2, Pt.4, Pt.10, Pt.11, Pt.12, Pt.13, and Pt.16), Neg; serum from non-allergic donor confirmed to have negative SPT results with CD extract. The arrows indicate the IgE-binding protein of CD and ZM crude extracts.

IgE reactivity of Zoy m 1 and Cyn d 1 was demonstrated in **Figure 2A**.

The immunoblot inhibition assay showed that the IgE binding to allergens in the *C. dactylon* crude extract was largely inhibited when sera were preincubated with 200 µg/ml of Cyn d 1 or 500 µg/ml of Zoy m 1 (**Figure 2B**). The different recombinant protein concentrations were needed to achieve a comparable level of inhibition. As a negative control, rD7, a recombinant major allergen protein from mosquito was shown to have only background reactivity to IgE in CD-sensitized serum samples, and did not significantly inhibit IgE reactivity to *C. dactylon* crude extract.

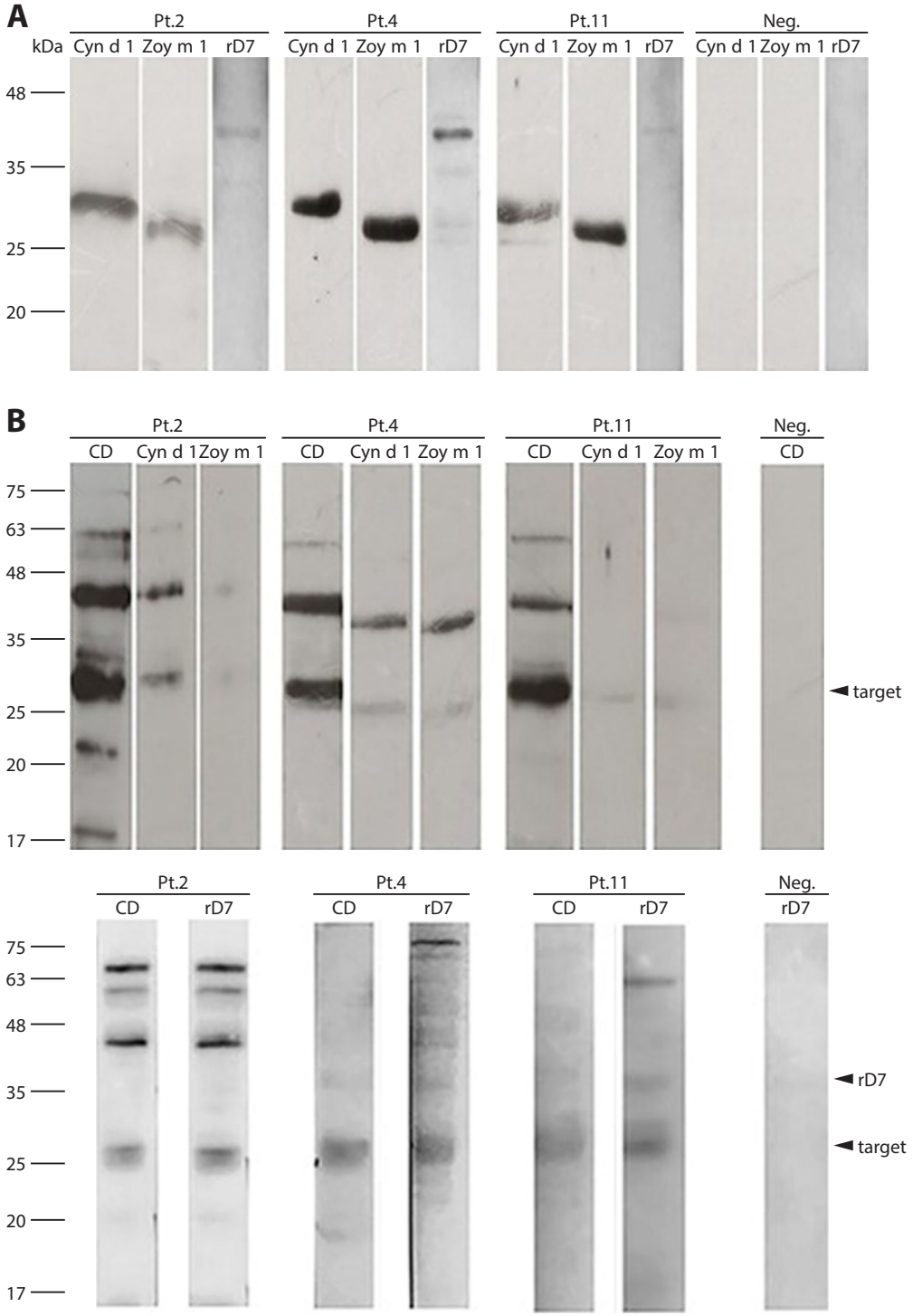


Figure 2. IgE-immunoblot profiles of beta-expansin recombinant proteins of Cyn d 1, Zoy m 1 and the irrelevant allergen Aedes aegypti (rD7) in three selected atopic patients (A). Note: serum of 3 individuals; Pt2, 4 and 11, Neg; serum of non-sensitized donor. Inhibition of specific IgE binding to protein in *C. dactylon* pollen extract using 200 µg/ml of Cyn d 1 or 500 µg/ml of Zoy m 1 (B). Note: lane CD = *C. dactylon* pollen extract without inhibition, lane Cyn d 1 and Zoy m 1 = *C. dactylon* pollen extract with inhibition using recombinant of Cyn d 1 and Zoy m 1, respectively, lane rD7 = *C. dactylon* pollen extract with inhibition using rD7, and N = serum of non-sensitized donor.

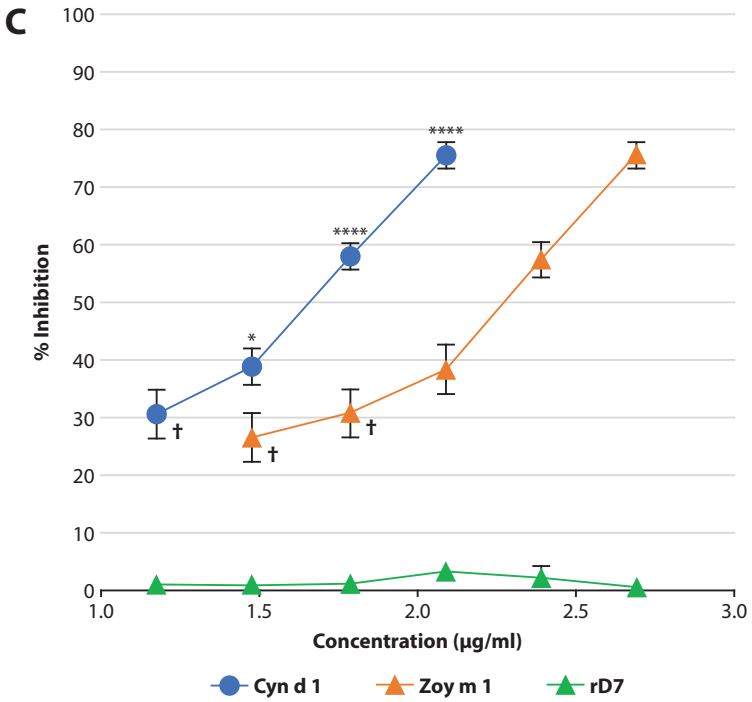


Figure 2. (Continued) Cross-inhibition of specific IgE binding to *C. dactylon* grass pollen proteins with Cyn d 1 and Zoy m 1 using sera from seven *C. dactylon* grass pollen allergic patients. Note: the rD7 from mosquito *A. aegypti* was used as a negative control (C). N = 7, except for data points marked with †, which represents n = 5 (without Pt.13 and Pt.16); * represents $p < 0.05$, ** represents $p < 0.0001$, error bars represent standard error of mean (SEM).**

To estimate the IgE binding potency of Cyn d 1 and Zoy m 1, ELISA inhibition assay was performed. The results showed that Cyn d 1 and Zoy m 1 were able to inhibit IgE binding to crude extract proteins from *C. dactylon* grass pollen at IC50 of 41.70 µg/ml and 154.40 µg/ml, respectively (**Figure 2C**). These results suggested that Zoy m 1 had substantial IgE reactivity and cross-reactivity to the major allergen in *C. dactylon* pollen, although with lower binding potency compared to Cyn d 1.

Clinical relevance of Z. matrella grass pollen sensitization

Due to the high possibility of *Z. matrella* pollen contributing to the grass pollen sensitization in Thai atopic patients, total pollen protein extract of *Z. matrella* (ZM), along with *C. dactylon* (CD), *S. halepense* (SH), *U. mutica* (UM), and *B. pertusa* (BP) were used for skin-prick test (SPT) at the ENT Allergy Clinic at Siriraj Hospital, Bangkok. Of the 168 total allergic rhinitis patients who underwent SPT procedure, 70 had a positive response to at least one grass pollen extract.

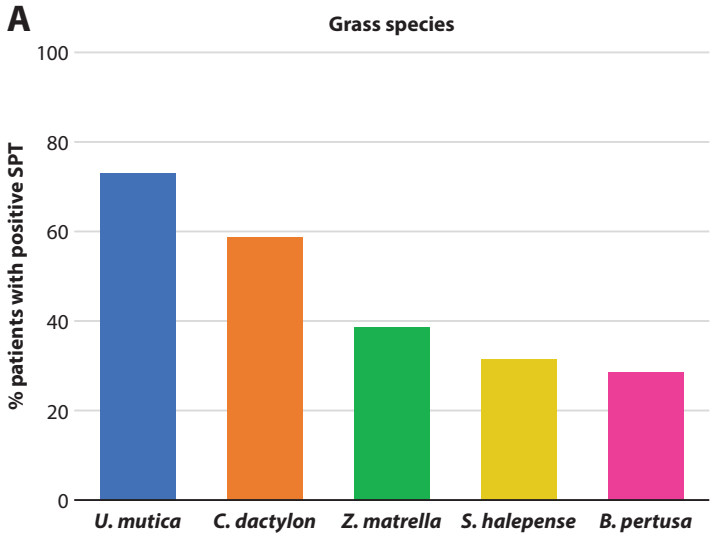


Figure 3. Skin-prick test (SPT) results from ENT Allergy clinic, Siriraj Hospital, Bangkok. The percentage of atopic patients with positive SPT results using five species of grass pollen (SPT was administered to 168 patients visiting the clinic between January to July 2016; the 70 patients who had positive SPT results with at least one grass pollen species were regarded as 100%) (A).

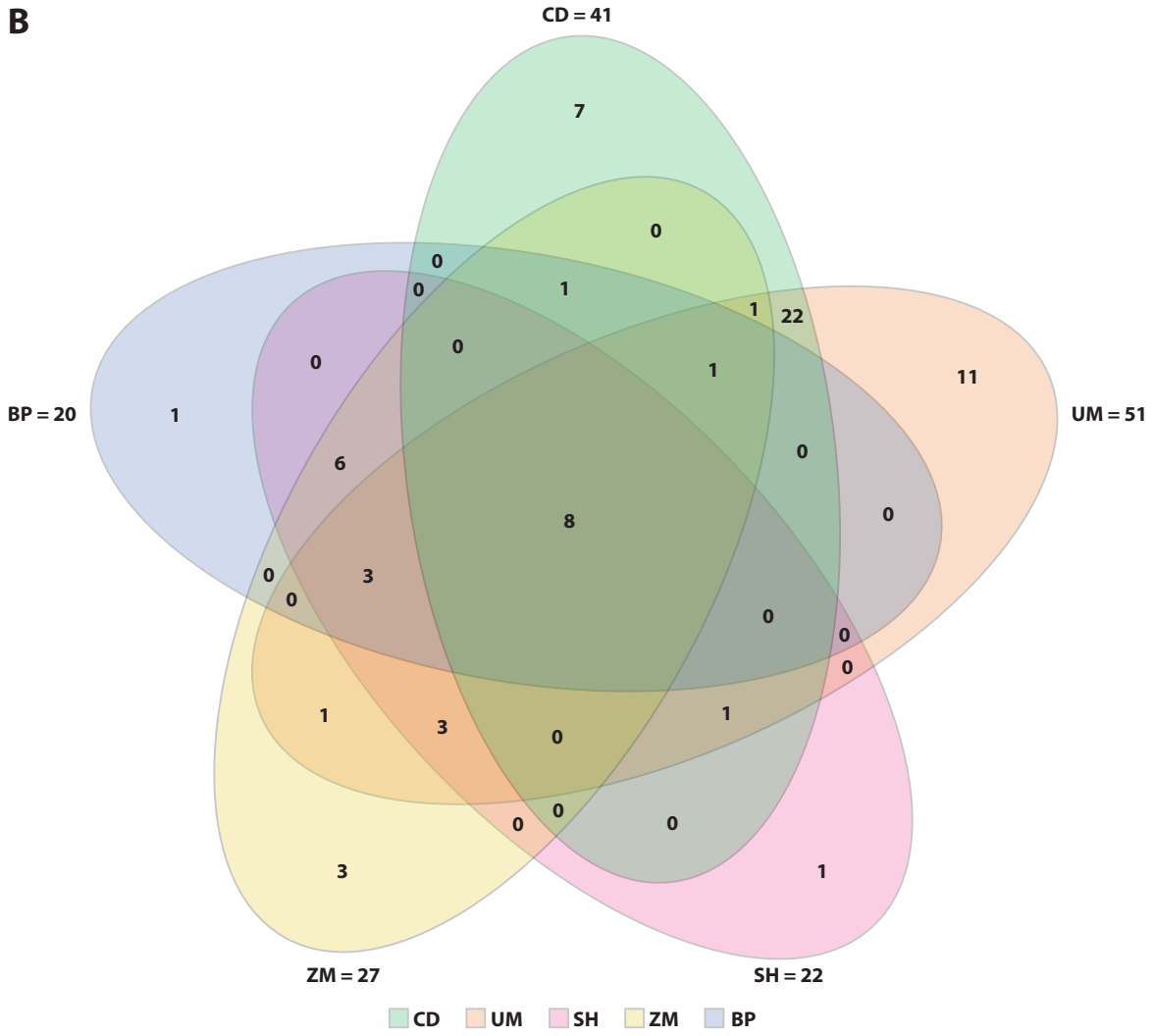


Figure 3. (Continued) Venn-diagram of prevalence of grass atopic patients in five grass pollen species. Note, UM: *U. mutica*, CD: *C. dactylon*, ZM: *Z. matrella*, SH: *S. halepense*, and BP: *B. pertusa* (B). Total N = 70.

Among these, the prevalence of sensitization was considerably high for *U. mutica* (UM) at 51/70 (73%) compared to *C. dactylon* (CD), *Z. matrella* (ZM), *S. halepense* (SH), and *B. pertusa* (BP) at 41/70 (59%), 27/70 (39%), 22/70 (31%), 20/70 (29%), respectively (**Figure 3A**). **Figure 3B** illustrates the overlap between sensitization of different grass pollen species. Eight patients had positive responses to all five species of grass pollen. Importantly, five patients showed positive response to only *Z. matrella* pollen extract. This Venn diagram demonstrates the clinical relevance of *Z. matrella* as an important source of allergenic pollen. Furthermore, patients sensitized to *Z. matrella* were more likely to also be sensitized to *U. mutica* (17/27, 63%) than *C. dactylon* (11/27, 41%). Based on the confirmed IgE reactivity of Zoy m 1 and positive SPT results, the beta-expansin of *Z. matrella* was confirmed as a major allergen and was named Zoy m 1.0101, based on the IUIS nomenclature.

Discussion

This study aimed to expand the knowledge about grass pollen sensitization in the tropical/subtropical regions. In particular, we examined the possible role of *Z. matrella* (Manila grass), a major turfgrass in these regions. Because the group-1 allergen from *Z. matrella* was highly similar to that of *C. dactylon*, we hypothesized that the immunoreactivity was also extremely similar. Thus, the cross-reactivity between these two allergens was examined. The results of ELISA and immunoblot inhibition assays revealed that Zoy m 1 could inhibit IgE binding to the natural proteins in the crude extract of *C. dactylon* pollen with slightly lower potency than Cyn d 1, suggesting extensive cross-reactivity. Cross-reactivity had been previously demonstrated for allergens with comparable level of similarity such as between between Pas n 1 of *P. notatum*, Cyn d 1 of *C. dactylon*, and Lol p 1 of *Lolium perenne*.[6,7] However, the serum IgE cross-reactivity between beta-expansins from Chloridoideae and Panicoideae subfamilies was incomplete and differences exist in the degree of amino acid similarity between beta-expansins of different grass subfamilies and between gene loci.[6,18]

Based on the high percent identity and cross-reactivity between group-1 allergens of *Z. matrella* and previously reported allergenic species, it is highly possible that *Z. matrella* pollen could elicit allergic reaction in atopic patients. In a previous study of the most common causative allergen of the ENT Allergy Clinic at Siriraj Hospital, Bangkok, in 2002 and 2004, 52.3% and 45.4% of 736 patients had a reaction to *C. dactylon* and *U. mutica* grass pollen, respectively.[9] The major allergen of these grass species is beta-expansin or group 1 allergen. The beta-expansin from *C. dactylon* showed the highest potential to produce allergic reaction and cross-reactivity to *S. halepense* and *U. mutica* grass pollen among Thai atopic patients.[13] However, sensitization to *Z. matrella* had never been reported previously. This study confirmed that a considerable number of grass pollen allergic patients had positive reactions to the *Z. matrella* pollen extract. In fact, a higher percentage of patients were sensitized to *Z. matrella* than *S. halepense*, which has long been considered an important allergen source in Thailand. Furthermore, a few patients appeared to be uniquely sensitized to *Z. matrella* and not the other four species tested in parallel. This result suggests that unique allergenic epitopes could be present in *Z. matrella* pollen, although it is also possible that these patients are in fact sensitized to other grass pollen species but the reaction was not strong enough to elicit a positive result on the skin-prick test due to the imprecise nature of the test. The result could be confirmed using more sensitive methods such as intradermal testing, which can be done in further studies.

Taken together, this study confirms the significance of *Z. matrella* as a source of allergenic pollen in tropical/subtropical regions. Interestingly, *C. dactylon* has been the only grass species from the Chloridoideae subfamily with extensive information about the allergenic protein. This study positions *Z. matrella* and its group-1 pollen allergen Zoy m 1 as an additional option for studies of allergens from the Chloridoideae subfamily.

Owing to the global climate changes, the distribution of subtropical grasses has extended both geographically and temporally, causing an increase in allergenic pollen production.[19] Thereby, the potential of human exposure to subtropical grass pollen also increases and extensively contributes to the burden of allergic diseases in parts of warming regions.[20,21] *Z. matrella* or Manila grass, along with other *Zoysia* spp., has become a major turfgrass in several Asia-Pacific countries, including Thailand, South Korea Japan and Australia.[22] It is also gaining popularity in the US due to its low-maintenance characteristics.[23] Although *Zoysia* spp. has been regarded as a species with low allergenicity in the past,[24,25] the increasing distribution warrants a public warning about its contribution to grass pollen allergy sensitization and cross-reactivity.

In conclusion, beta-expansin of *Z. matrella* can produce an allergic reaction in sensitized individuals and is cross-reactive with grass group-1 allergen from *C. dactylon* and potentially other subtropical grasses in Chloridoideae and Panicoideae subfamilies. Patients with grass pollen allergy should avoid the areas with *Z. matrella* or protect themselves from exposure to its pollen.

Acknowledgements

We are thankful to the staff of Rhinology & Allergy Division, Department of Otorhinolaryngology, Faculty of Medicine Siriraj Hospital, Mahidol University for the help with skin prick test and serum sample collection. We appreciate Dr. Thomas N. Stewart for proofreading this manuscript.

References

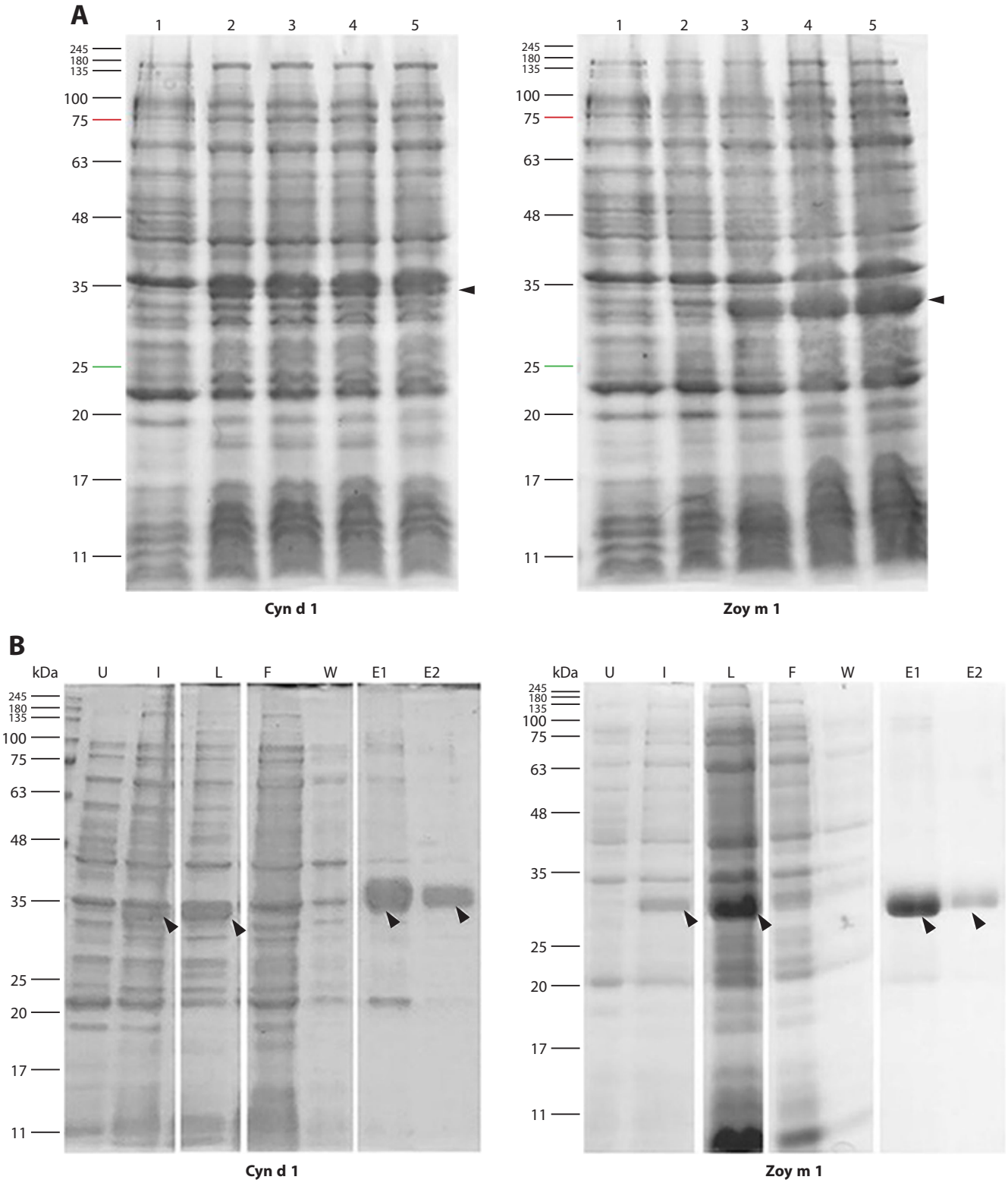
1. Weber RW. Cross-reactivity of pollen allergens. Current allergy and asthma reports. 2004;4(5):401-8.
2. Davies JM, Bright ML, Rolland JM, O'Hehir RE. Bahia grass pollen specific IgE is common in seasonal rhinitis patients but has limited cross-reactivity with Ryegrass. Allergy. 2005;60(2):251-5.
3. Duffort O, Quintana J, Ipsen H, Barber D, Polo F. Antigenic similarity among group 1 allergens from grasses and quantitation ELISA using monoclonal antibodies to Phl p 1. Int Arch Allergy Immunol. 2008; 145(4):283-90.
4. Timbrell VL, Riebelt L, Simmonds C, Solley G, Smith WB, McLean-Tooke A, et al. An immunodiagnostic assay for quantitation of specific IgE to the major pollen allergen component, Pas n 1, of the subtropical Bahia grass. Int Arch Allergy Immunol. 2014;165(4):219-28.
5. Tiwari R, Bhalla PL, Singh MB. Evaluation of molecular Basis of cross reactivity between rye and Bermuda grass pollen allergens. Allergol Int 2009;58(4):557-64.
6. Davies JM, Dang TD, Voskamp A, Drew AC, Biondo M, Phung M, et al. Functional immunoglobulin E cross-reactivity between Pas n 1 of Bahia grass pollen and other group 1 grass pollen allergens. Clin Exp Allergy. 2011;41(2):281-91.
7. Davies JM, Li H, Green M, Towers M, Upham JW. Subtropical grass pollen allergens are important for allergic respiratory diseases in subtropical regions. Clin Transl Allergy. 2012;2(1):1-10.
8. Visitsunthorn N, Visitsuntho K, Pacharn P, Jirapongsananuruk O, Bunnag C. Comparison of the efficacy and safety of pollen allergen extracts using skin prick testing and serum specific IgE as references. Asian Pac J Allergy Immunol. 2017;35(4):191-5.
9. Bunnag C, Jareoncharsri P, Tantilipikorn P, Vichyanond P, Pawankar R. Epidemiology and current status of allergic rhinitis and asthma in Thailand -- ARIA Asia-Pacific Workshop report. Asian Pac J Allergy Immunol. 2009;27(1):79-86.
10. CABI. *Zoysia matrella*. In: Invasive Species Compendium. Wallingford, UK: CAB International. 2019;(retrieved 10 October 2019).
11. Aud-In S, Somkid K, Songnuan W. Group-1 grass pollen allergens with near-identical sequences identified in species of subtropical grasses commonly found in Southeast Asia. Medicina (Kaunas). 2019;55(5).
12. Opasawatchai, A., Yolwong, W., Thuncharoen, W., Inrueangsri, N., Itsaradisaikul, S., Sasisakulporn, C., Jotikasthira, W., Matangkasombut, O., Reamtong, O., Manuyakorn, W., Songnuan, W., & Matangkasombut, P. (2020). Novel salivary gland allergens from tropical mosquito species and IgE reactivity in allergic patients. World Allergy Organization J, 13(2), 100099.
13. Pacharn P, Songnual W, Siriwatanakul U, Thongngarm T, Reamtong O, Jirapongsananuruk O, et al. Beta-Expansin of Bermuda, Johnson, and Para grass pollens, is a major cross-reactive allergen for Allergic Rhinitis patients in subtropical climate. Asian Pac J Allergy Immunol. 2018.
14. Smith PM, Suphioglu C, Griffith IJ, Theriault K, Knox RB, Singh MB. Cloning and expression in yeast *Pichia pastoris* of a biologically active form of Cyn d 1, the major allergen of Bermuda grass pollen. J Allergy Clin Immunol. 1996;98(2):331-43.
15. Chang, Peng, Lee, Chen, Chua, Tsai, et al. Sequence polymorphism of the group 1 allergen of Bermuda grass pollen. Clin Exp Allergy. 1999;29(4):488-96.
16. Au LC, Lin ST, Peng HJ, Liang CC, Lee SS, Liao CD, et al. Molecular cloning and sequence analysis of full-length cDNAs encoding new group of Cyn d 1 isoallergens. Allergy. 2002;57(3):215-20.
17. Yuan HC, Wu KG, Chen CJ, Su SN, Shen HD, Chen YJ, et al. Mapping of IgE and IgG4 antibody-binding epitopes in Cyn d 1, the major allergen of Bermuda grass pollen. Int Arch Allergy Immunol. 2012;157(2):125-35.
18. Campbell BC, Gilding EK, Timbrell V, Guru P, Loo D, Zennaro D, et al. Total transcriptome, proteome, and allergome of Johnson grass pollen, which is important for allergic rhinitis in subtropical regions. J Allergy Clin Immun. 2015;135(1):133-42.

19. Morgan JA, LeCain DR, Pendall E, Blumenthal DM, Kimball BA, Carrillo Y, et al. C4 grasses prosper as carbon dioxide eliminates desiccation in warmed semi-arid grassland. Nature. 2011;476(7359):202-5.
20. Beggs PJ, Bennett CM. Climate change, aeroallergens, natural particulates, and human health in Australia: state of the science and policy. APJPH. 2011;23(2 Suppl):46S-53.
21. Ziska LH, Beggs PJ. Anthropogenic climate change and allergen exposure: The role of plant biology. J Allergy Clin Immunol. 2012; 129(1):27-32.
22. Huang X, Wang F, Singh R, Reinert JA, Engelke MC, Genovesi AD, et al. Construction of high-resolution genetic maps of *Zoysia matrella* (L.) Merrill and applications to comparative genomic analysis and QTL mapping of resistance to fall armyworm. BMC genomics. 2016;17:562-.
23. Patton AJ, Schwartz BM, Kenworthy KE. Zoysiagrass (*Zoysia* spp.) History, Utilization, and improvement in the United States: A Review. Crop Sci. 2017;57(supplement1):S-37-S-72.
24. Kosman E, Eshel A, Waisel Y. The 'Travelling Salesman Problem': A new approach for identification of differences among pollen allergens. Int Arch Allergy Immunol. 1997;112(4):371-7.
25. Lee J, Sun H-J, Lee H-Y. Biohazard surveillance of allergic contact dermatitis in genetically-modified Zoysia grasses using patch testing. Allergy Asthma Respir Dis. 2015;3(2):134-8.

```
Z. matralla          MASSSARRQAQLAAVAVLLSAMVGSALCEIGDKPGPNITATYNEEWQDAKATFYGSNPRGAAPDDDGGACGYKNVDKAPF  80
S. halepense         ................................................................................  80
C. dactylon          ----------M......V.AS...G.W.AM............GDK.L.........D........H.......D......  70
Clustral Consensus             ******:*::***.* * :************.::* *********:********.*******:******  63
                                        IgE&igG4                              IgE
Z. matralla          FGMTGCGNEPIFKDGLGCGSCFEIKCKEPAECSDKPVLIRITDKNYEHIAAYHFDLSGKAFGSMAKQGQEDNLRKAGELT  160
S. halepense         ....................Y........................................................  160
C. dactylon          D....................Y...........GE....K.......................A...K.E..K.......M  150
Clustral Consensus    ****************** *:***********.:****:**********************:***:*:**:*******   139
                                  IgG4          IgE&IgG4     IgE&IgG4               IgG4
Z. matralla          LQFRRVKCEYPSKTKITFHVEKGCNDNYLALLVEYAAGDGDIVAVDIKPKGSDEFLPMKPSWGAIWRIDPKKPLKGPFTV  240
S. halepense         ................................................................................  240
C. dactylon          ............D..........SSP.......K......N..G.........V.....L.......M..P........I  230
Clustral Consensus   ************.**********.. *******:******:**.********* ***** *******:** ********:  211

Z. matralla          RLTSESGAKLVQEDVIPADWKPNTAYTSNLQY 272   100.00
S. halepense         ................................ 272    99.63
C. dactylon          .......GHVE......E....D.V.K.KI.F 262    81.68
Clustral Consensus   *******.:: ****** ****:*.*.*::*: 238   %identity
```

Supplemental Figure 1. The deduced amino acid sequences and alignment of beta-expansin from *Z. matrella*, *S. halepense*, and *C. dactylon*. Dots represent amino acids that were identical to beta-expansin from *Z. matrella*. Bold letters indicate predicted signal peptide. Underlines mark the IgE and IgG4 binding epitopes predicted based on beta-expansin sequence from *C. dactylon*.[14,15,16,17] The amino acid sequences were obtained from GenBank with accession numbers as follows: *Z. matrella* (QCX36431.1), *S. halepense* (QCX36434.1), and *C. dactylon* (AAK96255.1).



Supplemental figure 2. Expression of recombinant beta-expansin protein in Rosetta™ 2 (DE3)/pET-28a (+) system. The induction of (A) recombinant proteins from *C. dactylon* (Left) and *Z. matrella* (Right) were performed using a different IPTG concentration at 0.4 mM and 1 mM, and a different time at 4 and 6 hours. The proteins from soluble and insoluble (inclusion bodies) fractions were analyzed by 14% SDS–PAGE. Arrows indicate His-tagged beta-expansin protein band. Lane 1: uninduced cell; Lane 2-3 were induced with IPTG at 0.4 mM for 4 and 6 hours, respectively, and Lane 4-5 were induced with IPTG at 1 mM for 4 and 6 hours, respectively. (B) Purification of recombinant beta-expansin by Ni-NTA His.Bind® resin. Proteins were visualized by SDS-PAGE analysis (Left: Cyn d 1 from *C. dactylon*, Right: Zoy m 1 from *Z. matrella*) (B). U: uninduced, I: induced, L: cell lysate, F: flow-through, W: wash, E1-E2: eluted fractions. Arrows indicate His-tagged beta-expansin protein bands.