

Letter to the Editor

Identification and gene cloning of a new major allergen Cha o 3 from *Chamaecyparis obtusa* (Japanese cypress) pollen

To the Editor:

Trees of the Cupressaceae family produce many types of pollen. As typified by mountain cedar, these pollens cause pollinosis worldwide.¹ In Japan, the Cupressaceae family member *Chamaecyparis obtusa* (Japanese cypress) has been replanted over a substantial area.² Japanese cypress pollinosis is one of the most common allergic diseases, and the increasing number of patients with Japanese cypress pollinosis will remain a concern in the near future.

Cha o 1 and Cha o 2 are major allergens of Japanese cypress pollen, and they exhibit considerable amino acid sequence homology with the major Japanese cedar pollen allergens Cry j

1 and Cry j 2, respectively.^{3,4} Consistent with this sequence homology, cross-reactivity with some T-cell epitopes and IgE has been demonstrated.^{5,6} Because of their cross-reactivity, both Cry j- and Cha o 1-specific T_H2 responses are reportedly inhibited in patients who have received allergen-specific immunotherapy (ASIT) using standardized Japanese cedar pollen extract.² However, allergic symptoms and quality of life may worsen despite ASIT during Japanese cypress season, suggesting that unidentified allergens unique to Japanese cypress pollen exist.² In this study, we explored the third major allergen to be isolated from Japanese cypress pollen extract after Cha o 1 and Cha o 2.

On SDS-PAGE analysis of a crude extract of cypress pollen, we observed a diffuse major band (Cha o 3) at approximately 63 kDa (Fig 1, A). Cha o 3 was isolated using ion exchange chromatography (see the Methods section in this article's Online Repository at www.jacionline.org). Cha o 3 exhibited double bands (63 and

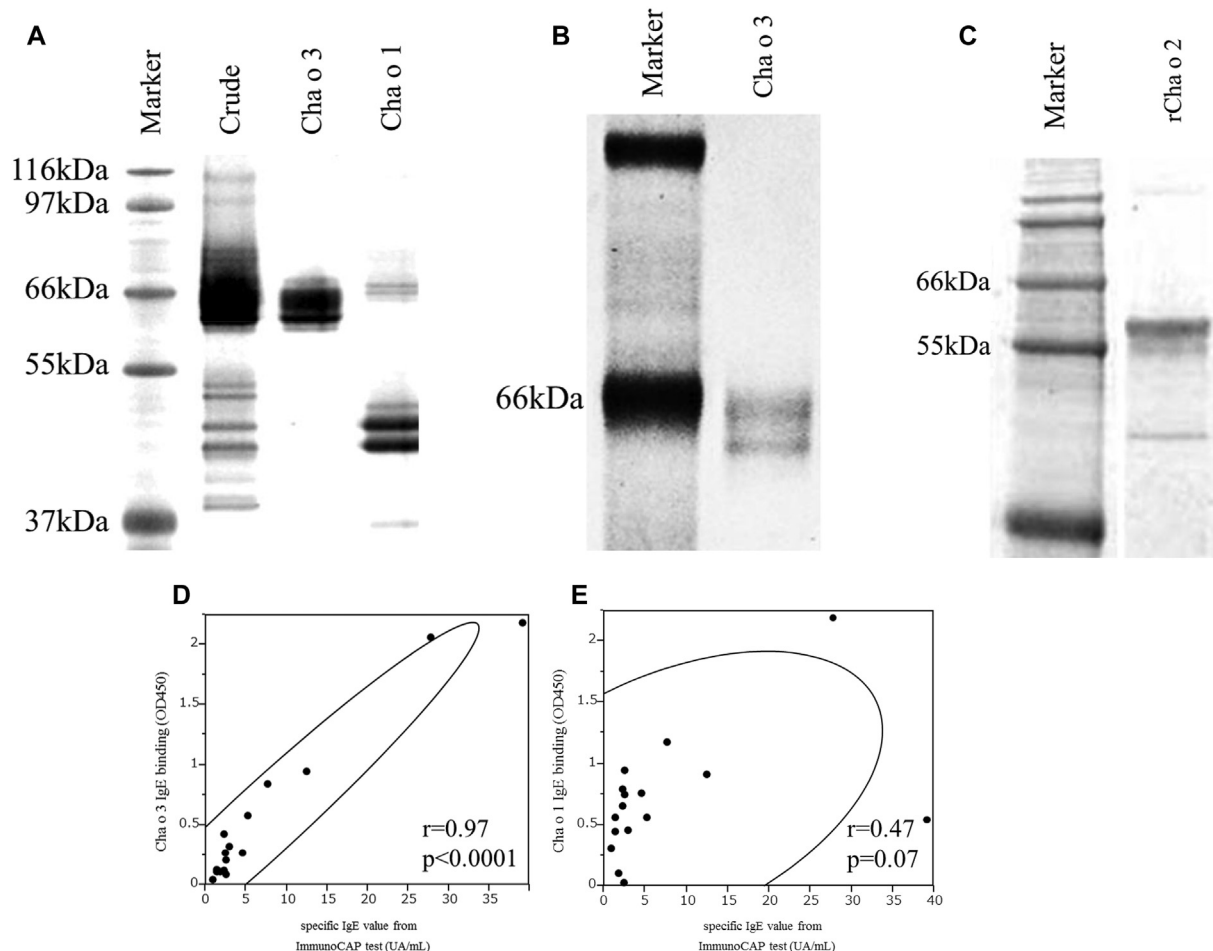


FIG 1. SDS-PAGE analysis and correlation between ImmunoCAP values and Cha o allergen-specific IgE. **A**, Cha o 3: purified Cha o 3 under nonreducing conditions, Cha o 1: purified Cha o 1 under nonreducing conditions. **B**, Purified Cha o 3 under reducing conditions. **C**, Purified recombinant Cha o 2. Because only a limited amount of Cha o 2 was obtained from the cypress extract, recombinant Cha o 2 was prepared. Correlation between ImmunoCAP values and Cha o 3-specific IgE binding measured by ELISA (**D**) or Cha o 1-specific IgE (**E**). Statistical analysis was performed using the Pearson correlation coefficient test. Lines indicate 95% probability ellipses. rCha o 2, recombinant Cha o 2.

66 kDa) under both nonreducing and reducing conditions (Fig 1, A and B). The Cha o 3 double bands were periodic acid-Schiff-stain positive, and the same peptide fragments were detected by LC-MS/MS from digests of each band (data not shown). Therefore, we concluded that the Cha o 3 double bands were the result of glycosylation difference, as with Cha o 1.³

To confirm whether Cha o 3 is an allergen in humans, an ELISA was performed to detect Cha o 3-specific IgE in the serum of 16 patients with Japanese cypress pollinosis. In addition to Cha o 1-specific IgE, a high frequency (14 of 16 patients [87.5%]) of Cha o 3-specific IgE binding was observed (see Table E1 in this article's Online Repository at www.jacionline.org). Surprisingly, a more significant correlation was observed between Cha o 3-specific IgE and Japanese cypress-specific IgE quantified by ImmunoCAP than between Cha o 1-specific IgE and Japanese cypress-specific IgE (Fig 1, D and E), suggesting that Cha o 3 is a previously undescribed Japanese cypress pollen allergen in humans that contributes significantly to diagnostic scores. In addition, basophil activation test results indicated that Cha o 3-specific IgE is functional in patients (see Fig E1 in this article's Online Repository at www.jacionline.org). A Cha o 3-dominant response was observed in patients with Japanese cypress pollinosis (Fig E1, C), suggesting that Cha o 3 is involved in the pathogenic mechanism of Japanese cypress pollinosis along with Cha o 1.

A lymphocyte stimulation test was used to elucidate the details of the pathogenesis of Cha o 3. PBMCs obtained from 16 patients showed a proliferative response to Cha o 3 of the same or greater magnitude than to other cypress allergens (Fig 2). Furthermore, the ratio of Cha o 3-specific T cells among proliferating lymphocytes was higher than that observed with other Japanese cypress allergens.

Molecular cloning of the Cha o 3 gene was also carried out (see the Methods section). The complete deduced amino acid sequence of the gene product comprised 556 residues, with a calculated molecular weight of 61,636 Da (see Fig E2 in this article's Online Repository at www.jacionline.org). The sequence from Met¹ to Ser²⁸ likely represents a signal peptide based on the *in silico* prediction. In addition, completely consistent results were observed by N-terminal amino acid sequence analysis (see Fig E2). Therefore, the estimated molecular weight of mature Cha o 3 is 58,865 Da. Cha o 3 did not exhibit any sequence identity with known Cry j or other Cha o allergens. A Pfam-A search indicated that Cha o 3 contains a cellulase (glycosyl hydrolase family 5) motif from Pro⁷⁰ to Gly³⁵³ (see Fig E3, A, in this article's Online Repository at www.jacionline.org). The deduced cellulase motif of Cha o 3 was highly conserved compared with other identified plant cellulases (see Fig E3, B). Because cellulolytic activity was detected in our preliminary study (data not shown), we concluded that Cha o 3 is a cellulase. Because the anthers of sweet pea reportedly contain large amounts of cellulase associated with pollen maturation,⁷ high expression of Cha o 3 in cypress pollen is reasonable. Our findings, therefore, suggest that proteins in the cellulase family may be novel allergens in the pollen of other plants, particularly trees of the Cupressaceae family.

Finally, to confirm the cross-reactivity of Cha o 3 with Japanese cedar allergen, we evaluated the production of IL-5, a typical T_H2 cytokine, in PBMCs isolated from another panel of patients with Japanese cedar pollinosis, with and without Japanese cedar-specific immunotherapy. As expected, Cry j 1-, Cry j 2-, and

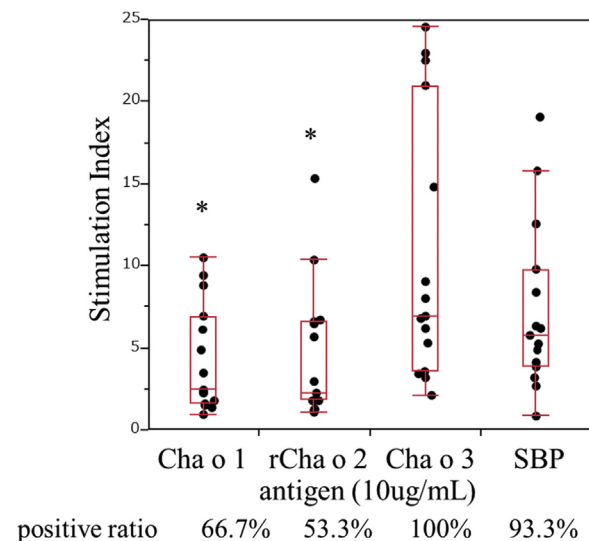


FIG 2. Proliferative response of PBMCs. PBMCs were stimulated with antigen (10 μ g/mL) for 3 days. After incubation for an additional 16 hours with [³H]-thymidine, [³H]-thymidine incorporation was measured. Statistical significance versus Cha o 3 was evaluated using Dunnett test (**P* < .01). The complementary DNA sequence of Cha o 3 was deposited in the GenBank sequence database under accession number HV942721. rCha o 2, recombinant Cha o 2; SBP, Sugi basic protein.

Cha o 1-associated IL-5 production was significantly suppressed in patients who received ASIT (see Fig E4 in this article's Online Repository at www.jacionline.org; 96.0%, 97.5%, and 92.5% inhibition compared with ASIT-naïve patients, respectively). Interestingly, Cha o 3-associated IL-5 production was also inhibited in patients who received ASIT (67.5% inhibition). However, Cha o 3 induced the highest level of IL-5 secretion among the 5 allergens in patients who received ASIT, suggesting that ASIT using Japanese cedar extract might not completely control Cha o 3-specific T_H2 responses.

These data raise 2 important points. First, it is unlikely that Cha o 3 is a unique allergen in Japanese cypress pollen due to the deduced presence of Cha o 3 homologs that are predicted to cross-react with Cha o 3 in Japanese cedar pollen, similar to the relationship between Cry j 1 and Cha o 1. Second, the levels of Cha o 3 homologs in Japanese cedar pollen extract may not be sufficient for desensitization, or their homology may be low. It is noteworthy that Cupressaceae pollens share allergenic components. For example, Cha o 1 has high (>85%) sequence identity to Jun a 1 (from mountain cedar) and Jun v 1 (from eastern red cedar).^{8,9} Further experiments aimed at identifying Cha o 3 homologs in cedar pollen are needed to enhance understanding of Japanese cedar and cypress pollinosis.

In conclusion, we demonstrated that Cha o 3 is a novel major allergen of Japanese cypress pollen. The allergen was designated Cha o 3 according to WHO/IUIS Allergen Nomenclature Subcommittee recommendations. New ASIT approaches using Cha o 3 in addition to standardized Japanese cedar extract may be useful for improving the efficacy of ASIT throughout the cypress pollen season. Moreover, the present results suggest the presence of a previously undescribed allergen in Japanese cedar pollen. We hope the discovery of Cha o 3 will contribute to the development of basic research and approaches for treating Japanese cedar, cypress, and *Cupressaceae* pollinosis.

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METHODS

Plant materials

Pollen from Japanese cypress was purchased from the Japan Forest Tree Breeding Association (Tokyo, Japan). Female flowers were collected from Japanese cypress trees located in Hanno-shi Saitama, Japan, immediately before the pollen season. Pollen and female flowers were stored at -80°C before use.

Subjects

Patients were selected from the employees of Hanno Research Center, Taiho Pharmaceutical Co, Ltd, on the basis of clinical symptoms of seasonal allergic rhinitis suffered every spring. All serum samples were measured for Japanese cedar pollen and Japanese cypress pollen-specific IgE using ImmunoCAP (at the clinical test service of SRL, Inc, Tokyo, Japan). Finally, we enrolled 16 patients who were determined to be specific IgE seropositive (at least ImmunoCAP class 2, $\geq 0.71 \text{ U}_A/\text{mL}$) to both kinds of pollen. All studies were approved by the Institutional Ethical Review Board for basic research. For the determination of allergen-specific IL-5 production by PBMCs, 47 patients who were diagnosed with Japanese cedar and Japanese cypress pollinosis by the Department of Otolaryngology-Head & Neck Surgery, Okayama University Hospital, were enrolled in this study after they provided informed consent. A total of 24 of 47 patients received subcutaneous ASIT using a standardized extract of Japanese cedar pollen (Torii Pharmaceutical Co Ltd, Tokyo, Japan) over a period of at least 2 years, as described in a previous report.^{E1}

Isolation of native Cha o 1 and Cha o 3 protein from Japanese cypress pollen

Approximately 5 g of Japanese cypress pollen was homogenized in 25 mL of neutral buffer (10 mM Tris-HCl, pH 7.8) using a Teflon homogenizer. After incubation for 1 hour at 4°C , this crude extract was centrifuged at $12,000g$ for 20 minutes at 4°C . The supernatant was then subjected to Vivapure Q anion exchange chromatography (Sartorius Stedim, Göttingen, Germany), and the flow-through was collected. This unadsorbed fraction was dialyzed against 10 mM acetate buffer (pH 5.0) and applied to a Vivapure S cation exchange column (Sartorius Stedim) equilibrated in the same 10 mM acetate buffer (pH 5.0) to adsorb Cha o 3 onto the carrier resin. Adsorbed Cha o 3 was eluted by gradient elution using NaCl solution in the neutral buffer described above. Cha o 3 was eluted in neutral buffer containing 0.5 mol/L NaCl. Cha o 1 was isolated from crude extract of Japanese cypress pollen using a previously reported method.^{E2} Contamination of LPS in the purified Cha o 1 and Cha o 3 samples was examined by using the Limulus Amebocyte lysate Pyrosta ES-F single test (Wako Pure Chemical, Osaka, Japan; detection limit is 0.015 EU/mL) and the levels were not detectable and were less than 1 EU/mL.

Expression of recombinant Cha o 2 protein

Because the amount of Cha o 2 from cypress extract was not sufficient for use in the various experiments, recombinant Cha o 2 (rCha o 2) was prepared according to a previous report.^{E3,E4} Briefly, the Cha o 2 complementary DNA (cDNA) fragment was synthesized and subcloned into pENTR4 vector (Life Technologies, Waltham, Mass) through *Bam*HI and *Xho*I sites. The subcloned Cha o 2 cDNA was recombined into pEXP1-DEST expression vector using Gateway LR Clonase enzyme mix. The resulting Cha o 2 expression vector was transformed into *Escherichia coli* (BL21 STAR, Life Technologies) for protein expression. Expression of rCha o 2 was induced in 0.6 mM isopropyl- β -D-1-thiogalactopyranoside for 6 hours. After the lysis of *E. coli* cells, rCha o 2 in the lysate was purified using Ni^{2+} NTA resin. The purity and molecular weight of rCha o 2 was confirmed by SDS-PAGE. The presumed molecular weight of rCha o 2 was approximately 59 kDa because of the inclusion of 6 $\times$ His, Xpress tag, and spacer peptides in the pEXP1-DEST vector in addition to rCha o 2. Contamination of LPS in the rCha o 2 sample was not detected.

Characterization of purified Cha o 3 protein

Purified Cha o 3 was analyzed by SDS-PAGE using a Novex NuPAGE SDS-PAGE system (Life Technologies) and a general protocol. Gel staining was performed using SYPRO Ruby Protein Gel Stain (Lonza, Basel,

Switzerland), and protein bands were detected using ImageQuant LAS 4010 (GE Healthcare, Little Chalfont, United Kingdom). N-terminal and internal amino acid sequence analysis of purified Cha o 3 was conducted by Toray Research Center Inc (Tokyo, Japan) using a Procise 492cLC protein sequencer (Applied Biosystems, Foster City, Calif). The N-terminal sequence ($\text{NH}_2\text{-LPLLTRGRWVDGATGLRVKLAXVNWVGH}$ L, where X indicates an unidentified amino acid) and the internal amino acid sequence ($\text{NH}_2\text{-SPLISTNECICITDSDHCYP}$) were obtained (Fig E2).

Determination of human IgE binding by ELISA

Allergen-specific IgE binding was assessed by ELISA. Allergen solution (20 $\mu\text{g/mL}$) was incubated in a Maxisorp 96-well plate (Thermo Fisher Scientific, Waltham, Mass) overnight at 4°C to coat wells with allergens. After washing 5 times with wash buffer (1% BSA in PBS[$-$]/0.05% Tween 20), blocking buffer (1% BSA in PBS[$-$]) was added to each well and incubated for 1 hour at room temperature. After plate washing, 5-fold-diluted serum samples or negative control serum (human type AB serum, MP Biomedicals, Santa Ana, Calif) was added and incubated for 2 hours at room temperature. The plate was then washed 5 times, and 7000-fold-diluted horseradish peroxidase-labeled goat antihuman IgE antibody (Novus Biologicals, Littleton, Colo) was added as a secondary antibody and incubated for 40 minutes at room temperature. Substrate solution was prepared with TMB Substrate Reagent Set (BD Biosciences, San Jose, Calif) and added to each well. After 15 minutes of incubation, an equal volume of 2 mol/L H_2SO_4 solution was added to each well to stop the reaction. Finally, the optical density (OD) at 450 nm was measured using a microplate reader (Tecan, Männedorf, Switzerland). Specific IgE binding was defined as positive when the OD_{450} value was more than 2 times the OD_{450} value of the negative control.

Basophil activation test

Heparinized whole blood was analyzed using an Allergen Kit (Beckman Coulter, Brea, Calif) according to the manufacturer's instructions. Briefly, indicated concentrations of allergen solutions and 20 μL of an antibody cocktail composed of antihuman CD3-PC7, antihuman CRTH2-fluorescein isothiocyanate (FITC), and antihuman CD203c-phycoerythrin (PE) were added to 100 μL of heparinized whole blood. The samples were immediately incubated in a 37°C water bath for 15 minutes under shaded light. Next, 100 μL of stop solution and 2 mL of "Fix-and-Lyse mixture" were added to each sample and incubated at room temperature for 10 minutes. Cells were collected by centrifugation and analyzed using a BD FACSCanto flow cytometry system (BD Biosciences).

Cloning of Cha o 3 cDNA

Total RNA was isolated using PureLink Plant RNA Reagent (Life Technologies) from the female flower of Japanese cypress. Subsequently, cDNA was synthesized using a Superscript III First-Strand Synthesis System for RT-PCR (Life Technologies). According to the results of Cha o 3 protein sequencing, highly conserved regions were observed in an unnamed protein product of *Vitis vinifera* (accession no. CAO65892). Hence, we designed forward (5'-AGAGTAAAGCTAGCATGCGTCAATTGG-3') and reverse (5'-CAGCACAGCACCCTTGGGCTTACTCAC-3') primers on the basis of this information. After PCR amplification, the sequence of the PCR product was determined using a DNA sequencer (3130xl; Applied Biosystems). The second forward (5'-CTGGTGTGTCATGGTCATATTG-3') and reverse (5'-GAGTCAGTGATGCAIATRCAYTC-3') primers were designed on the basis of identified cDNA sequence and information for the similar protein in *V. vinifera*, respectively. PCR amplification and sequencing of the cDNA were performed again to determine the internal sequence of the Cha o 3 cDNA. Subsequently, the 5' and 3' regions of the Cha o 3 gene were identified by 5'- or 3'-rapid amplification of complementary DNA ends (RACE) using a GeneRacer kit (Invitrogen, Waltham, Mass) and a 5'/3' RACE kit (Roche, Basel, Switzerland). 5'-RACE was performed using the primers 5'-CTCCGG ATTGTTGTGCTCGATTTC-3' and 5'-CAGCACAGCACCCTTGGGCTT ACTCAC-3'. 3'-RACE was performed using the primer 5'-GTTTCATCTG CATTTCACCTCAAGG-3'.

***In silico* analysis of the Cha o 3 protein**

Homology was assessed by searching the Protein Sequence Data Banks using NCBI-BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The signal peptide was predicted using GENETYX software, ver. 9.0.2. A Pfam-A search was conducted to predict the family of protein Cha o 3 (<http://pfam.xfam.org/search>).

Quantification of allergen-specific IL-5 production by PBMCs

PBMCs were separated by Ficoll-Paque density gradient centrifugation from whole-blood samples obtained from 47 patients in the spring, as described previously.^{E5} PBMCs (1×10^6) were stimulated with 10 $\mu\text{g/mL}$ of Cry j 1, Cry j 2 (Hayashibara Co, Ltd, Okayama, Japan), Cha o 1, rCha o 2, or Cha o 3. After 72 hours of culturing, the supernatant was collected and stored at -80°C until cytokine analysis. Finally, the presence of allergen-specific IL-5 in the supernatant was measured using an IL-5 ELISA kit (R&D Systems, Minneapolis, Minn).

Lymphocyte stimulation test

PBMCs (2×10^5) were stimulated with 10 $\mu\text{g/mL}$ of Cha o 1, rCha o 2, Cha o 3, or Sugi (Japanese cedar) basic protein (Hayashibara Co., Ltd) for 72 hours. Incorporation of [^3H]-thymidine (PerkinElmer, Waltham, Mass) was assessed using a standard method, as described previously.^{E5} The results are shown as the stimulation index (SI), where $\text{SI} = (\text{mean count per minute [cpm] in triplicate cultures stimulated with antigen})/(\text{mean cpm in unstimulated$

triplicate cultures). An SI value of more than 2 was set as a positive response. Expected levels of contaminated LPS in the Cha o 1 and rCha o 2 in the assay system were undetectable and that in the Cha o 3 was less than 0.05 EU/mL. It was confirmed that these levels of LPS did not induce lymphocyte stimulation in this system.

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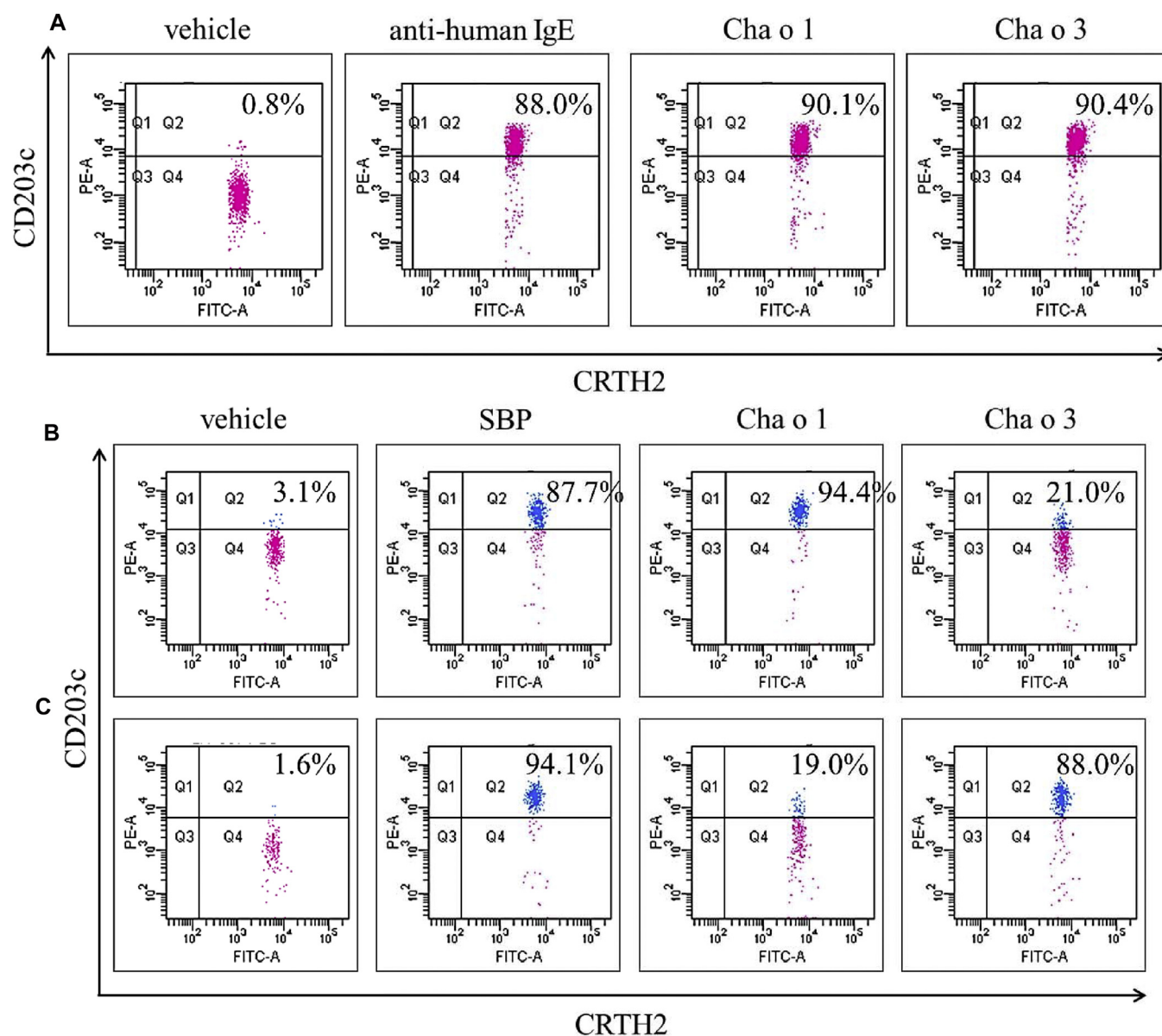


FIG E1. Cha o 3 induces basophil activation in patients with Japanese cypress pollinosis. **A**, Whole blood of V043 was stimulated with antigen (0.1 μ g/mL) or anti-human IgE (10 μ g/mL) as a positive control for 15 minutes at 37°C. Whole blood of V016 (**B**) or V037 (**C**) was stimulated with each allergen (1 ng/mL). *CRTH2*, Chemoattractant receptor-homologous molecule on Th2 cells; *SBP*, Sugi basic protein.

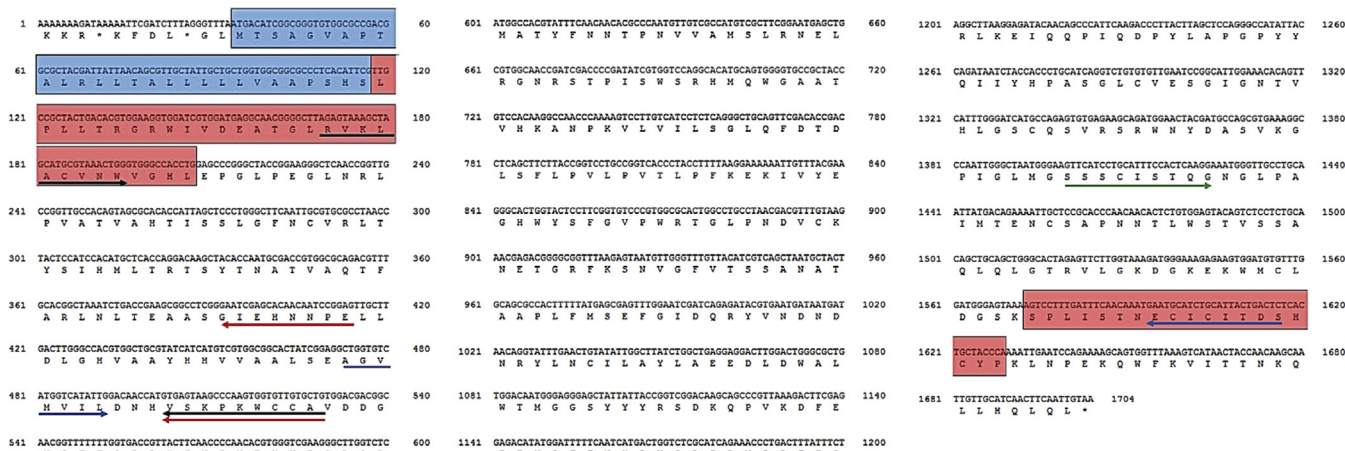


FIG E2. Sequence of cloned cDNA of Cha o 3 and deduced amino acid sequence. Domains to which primers were applied for PCR cloning are shown by *arrows* (*black arrows*, first primers; *blue arrows*, second primers; *red arrows*, primers for 5'-RACE; *green arrow*, primer for 3'-RACE). Arrow direction indicates forward or reverse primers. The *blue box* represents the predicted signal peptide. Peptides that were identified by amino acid sequence analysis are shown in *red boxes*. Several independently isolated cDNA clones identified polymorphisms at nucleotides 88 (C or A), 264 (A or C), 681 (C or T), 772 (G or A), 792 (A or G), 870 (C or T), 895 (G or A), and 1029 (G or A). Nearly half the mutations encoded the same amino acids, except nucleotides 88 (Pro or Thr), 772 (Val or Ile), and 895 (Val or Ile). Because of these polymorphisms, the most frequent cDNA clone is discussed in this report.

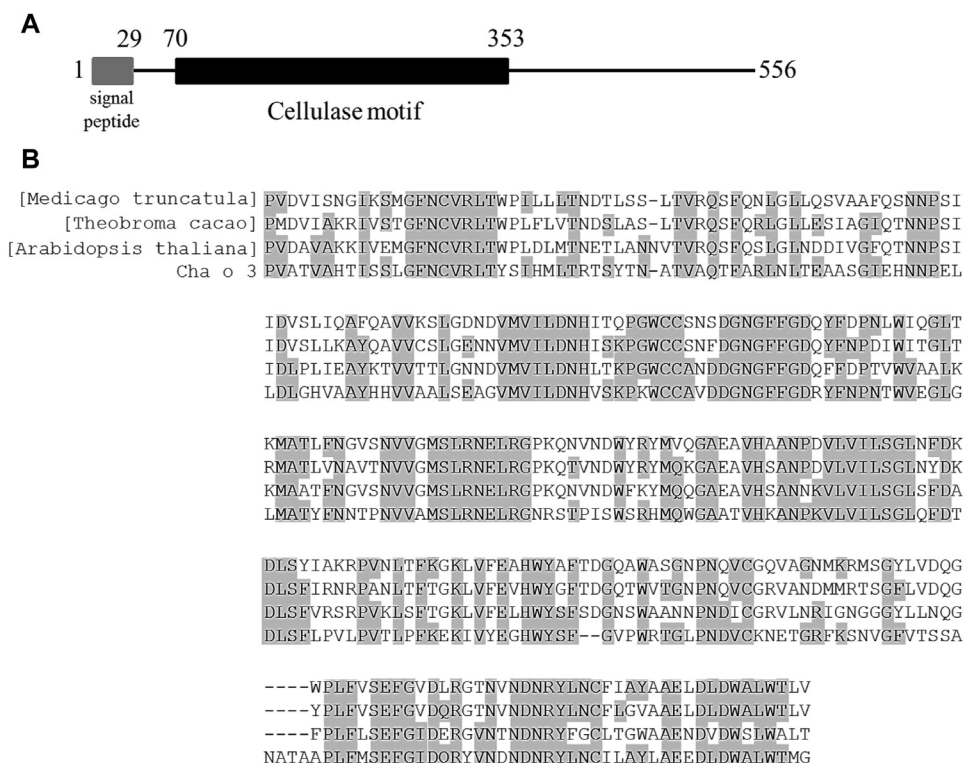


FIG E3. A, Predicted Cha o 3 protein. **B,** ClustalW multiple sequence alignment for homology analysis. The predicted cellulase domain of Cha o 3 (from Pro⁷⁰ to Gly³⁵³) was compared with the cellulases of *Medicago truncatula* (accession no. KEH20742), *Theobroma cacao* (accession no. XP_007044872), and *Arabidopsis thaliana* (accession no. NP_172772). Shaded areas indicate consistent amino acid sequence compared with Cha o 3.

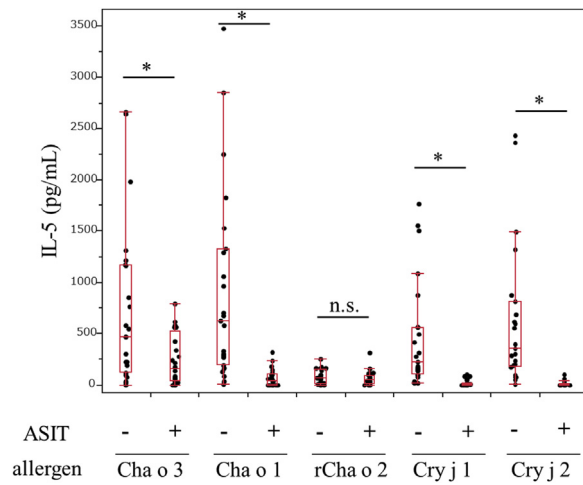


FIG E4. IL-5 production by PBMCs collected from patients with Japanese cedar and cypress pollinosis with or without Japanese cedar-specific immunotherapy. PBMCs were stimulated with 10 μ g/mL of the indicated allergens for 72 hours. The IL-5 content of the supernatant was determined by ELISA. Statistical analyses were performed using the 2-tailed Student *t* test (**P* < .01).

TABLE E1. Levels of allergen-specific IgE antibody in patient serum samples*

ID	Japanese cedar pollen		Japanese cypress pollen		OD ₄₅₀	
	IgE (U _A /mL)	Class	IgE (U _A /mL)	Class	Cha o 1	Cha o 3
V016	100<	6	39.1	4	+	+
V037	100<	6	27.8	4	+	+
V031	73.6	5	12.5	3	+	+
V043	100<	6	7.63	3	+	+
V045	75.9	5	5.18	3	+	+
V014	36.1	4	4.58	3	+	+
V051	46.7	4	2.92	2	+	+
V026	33	4	2.6	2	+	+
V050	43.7	4	2.6	2	+	—
V047	31	4	2.41	2	—	+
V012	49.2	4	2.35	2	+	+
V042	34.2	4	2.34	2	+	+
V013	8.8	3	1.77	2	+	+
V018	19.5	4	1.43	2	+	+
V040	19.5	4	1.43	2	+	+
V021	21.6	4	0.88	2	+	—
Positive ratio					93.8%	87.5%

*Allergen-specific IgE binding was determined by ELISA. IgE binding against Cha o 1 or Cha o 3 was determined to be positive when the OD₄₅₀ value was more than twice that of negative control serum. Japanese cedar and cypress pollen-specific IgE were evaluated using the ImmunoCAP method.