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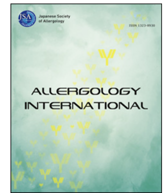

Original Article

Identification of Cha o 3 homolog Cry j 4 from *Cryptomeria japonica* (Japanese cedar) pollen: Limitation of the present Japanese cedar–specific ASIT

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cDNA, Complementary DNA; ASIT, Allergen-specific immunotherapy; BAT, Basophil activation test; RACE, Rapid amplification of cDNA ends; rCry j 4, Recombinant Cry j 4; PBMC, Peripheral blood mononuclear cell; TCL, T cell line; TCC, T cell clone; IL, Interleukin

A B S T R A C T

Background: About one-third of the Japanese population suffers from Japanese cedar pollinosis, which is frequently accompanied by Japanese cypress pollinosis. Recently, a novel major Japanese cypress pollen allergen, Cha o 3, was discovered. However, whether a Cha o 3 homolog is present in Japanese cedar pollen remains to be determined.
Methods: Western blot analysis was performed using Cha o 3–specific antiserum. In addition, cloning of the gene encoding Cry j 4 was conducted using total cDNA from the male flower of Japanese cedar trees. Allergen potency and cross-reactivity were investigated using a T-cell proliferation assay, basophil activation test, and ImmunoCAP inhibition assay.
Results: A low amount of Cha o 3 homolog protein was detected in Japanese cedar pollen extract. The deduced amino acid sequence of Cry j 4 showed 84% identity to that of Cha o 3. Cross-reactivity between Cry j 4 and Cha o 3 was observed at the T cell and IgE levels.
Conclusions: Cry j 4 was discovered as a counterpart allergen of Cha o 3 in Japanese cedar pollen, with a relationship similar to that between Cry j 1–Cha o 1 and Cry j 2–Cha o 2. Our findings also suggest that allergen-specific immunotherapy (ASIT) using Japanese cedar pollen extract does not induce adequate immune tolerance to Cha o 3 due to the low amount of Cry j 4 in Japanese cedar pollen. Therefore, ASIT using Cha o 3 or cypress pollen extract coupled with Japanese cedar pollen extract is required in order to optimally control allergy symptoms during Japanese cypress pollen season.
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Introduction

Many types of pollen from trees of the families *Cupressaceae*/*Taxodiaceae*, as typified by mountain cedar and Japanese cedar, are known to causes pollinosis worldwide.[1] In Japan, pollens of *Cryptomeria japonica* (Japanese cedar) and *Chamaecyparis obtusa* (Japanese cypress), which belong to the *Taxodiaceae* and *Cupressaceae* families, respectively, are major causes of allergic diseases. Large amounts of Japanese cedar pollen are dispersed between February and April, whereas Japanese cypress pollen is dispersed from March through early May. Therefore, various allergic symptoms such as allergic rhinitis and conjunctivitis diminish the quality of life (QOL) of Japanese cedar and Japanese cypress pollinosis patients for a substantial time in spring. In addition, because the amount of Japanese cedar and Japanese cypress pollen dispersed each year is increasing, there are concerns that the number of patients with Japanese cedar and Japanese cypress pollinosis will increase in the near future.[2]

Cross-reactivity between the pollen allergens of Japanese cedar and Japanese cypress is frequently discussed because 70% of patients with Japanese cedar pollinosis also experience allergic rhinitis caused by Japanese cypress pollen.[2] The major Japanese cedar pollen allergens Cry j 1[3,4] and Cry j 2[5,6] were identified and cloned and found to exhibit considerable amino acid sequence homology with the major Japanese cypress pollen allergens Cha o 1[7] and Cha o 2,[8] respectively. This sequence homology also suggested the potential for cross-reactivity for some T-cell epitopes and at the IgE level.[9–12] Thus, allergic symptoms can be evoked in Japanese cedar pollinosis patients following dispersion of Japanese

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cypress pollen. Similarly, many allergens produced by trees belonging to the *Cupressaceae* family and the closely related families *Taxodiaceae* and *Podocarpaceae* reportedly exhibit allergenic cross-reactivity.[1]

Although almost all Japanese cedar pollinosis patients receive symptomatic treatment consisting of a histamine H1–receptor antagonist, allergen-specific immunotherapy (ASIT) using a standardized extract of Japanese cedar pollen was developed to “cure” the Japanese cedar pollinosis.[13] However, allergic symptoms and QOL can decline despite strong inhibition of the Th2 response to known major allergens during Japanese cypress season,[13,14] suggesting that additional allergens unique to Japanese cypress pollen remain to be identified.

Recently, we reported the isolation and identification of a novel major allergen from Japanese cypress pollen: Cha o 3.[15] Interestingly, compared with Cry j 1 and Cha o 1, partial (but not significant) inhibition of Cha o 3–specific Th2 responses was observed in patients with Japanese cedar and cypress pollinosis who were treated with allergen-specific subcutaneous immunotherapy using Japanese cedar pollen extract, which is not expected to contain Cha o 3. Although these data were suggestive of the presence of a Cha o 3 homolog in Japanese cedar pollen, the pathologic mechanisms of Japanese cedar and Japanese cypress pollinosis remain unclear.

Therefore, the aim of the present study was to identify the novel Japanese cedar pollen allergen as a counterpart to Cha o 3. Cloning revealed that the novel Japanese cedar pollen allergen, Cry j 4, is a homolog of Cha o 3. Cross-reactivity between Cry j 4 and Cha o 3 was also characterized. The discovery of Cry j 4 highlights issues that must be considered in ASIT for Japanese cedar and cypress pollinosis using only Japanese cedar extract.

Methods

Plant materials

Male flowers were collected from Japanese cedar trees located in Inashiki-shi Ibaraki, Japan, immediately before the pollen season and stored at −80 °C prior to use.

Cloning of Cry j 4 cDNA

Total RNA was isolated from male flowers of Japanese cedar using PureLink® Plant RNA Reagent (Life Technologies, Carlsbad, CA, USA). cDNA was then synthesized from the total RNA using the Superscript III First-Strand Synthesis System for RT-PCR (Life Technologies). In order to design PCR primers, we searched the deduced nucleotide sequence encoding an identical peptide corresponding to Cha o 3 (NH_2-WIVDEATGLR-COOH) using ForestGEN (http://forestgen.ffpri.affrc.go.jp/en/index.html) against the cDNA library data for Japanese cedar. Two highly conserved consensus sequences (accession numbers: BY882225.1 and BY900252.1) were identified. Based on this information, we designed forward (5′-AGGTGGATCGTGGAYG-3′), (5′-TCAAGCTTAACTAGCCGTGGAYGAGGCAAC-3′) and reverse (5′-GTTGATGCAACAATTKYTTG-3′), (5′-TTAAACCTCGAGTGCGCAA-CAATTKYTTGTTGGT-3′) primer sets. After nested PCR amplification using KOD Fx neo (TOYOBO, Osaka, Japan), the PCR products were sequenced using a 3130xl DNA sequencer (Applied Biosystems). The 5′ and 3′ regions of the Cry j 4 gene were subsequently determined by rapid amplification of cDNA ends (RACE) using a SMARTer® RACE 5′/3′ kit (Clontech, Mountain View, CA, USA) and 2nd-generation 5′/3′ RACE kit (Roche, Basel, Switzerland), respectively. 5′-RACE was performed with the primer 5′-GCCTAACTGCAGCTGTCCAGAGGA-GACTG-3′, and 3′-RACE was performed with two primers: 5′-GATGGAAGTATGATGCCAGT-3′ and 5′-AGTGGATGTGTTTAGATGGG-3’. Finally, the complete cDNA sequence of Cry j 4 was confirmed by nested PCR using the following primers: forward, 5′-GATTTCATGR-CAGCGACAG-3’ & 5′-CATGRCAGCGACAGSGAT-3’; and reverse, 5′-TCAAAGTTGATGCAACAATTG-3’. Homology analysis using Protein Sequence Data Banks was performed with NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Expression of recombinant Cry j 4 (rCry j 4) protein

Because the amount of native Cry j 4 obtained from Japanese cedar was low, recombinant Cry j 4 was prepared using the silkworm-baculovirus expression system (ProCube) (Sysmex Corporation, http://procube.sysmex.co.jp/eng/) as described previously.[16] Briefly, the cDNA sequence of mature Cry j 4 was inserted into the pHS14 vector constructed to produce the fusion protein with a Flag (DYKDDDDK) tag at the C-terminus of mature Cry j 4. The resulting Cry j 4-Flag expression vector was transformed into the baculovirus and infected into a silkworm. Expressed rCry j 4 was then purified using DDDDK-tagged Protein Purification Gel (MBL, Aichi, Japan).

Western blotting

Cry j 4–specific antiserum was obtained by immunizing rabbits with 400 μg of rCry j 4 emulsified in complete Freund's adjuvant, with three subsequent booster immunizations with 200 μg of rCry j 4 emulsified in incomplete Freund's adjuvant (custom service conducted by Sigma–Aldrich, St. Louis, MO, USA). Pollen extract (Japanese cedar and Japanese cypress) and native Cha o 3 were prepared as described previously.[15] Pollen extract (isolated with 20 mM Tris–HCl, pH 9.0), pollen lysate (pollen grains were suspended in M-PER mammalian protein extraction reagent [Thermo Fisher Scientific, Waltham, MA, USA] and lysed by sonication), and native Cha o 3 were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using a Novex® NuPAGE® SDS-PAGE system (Life Technologies) according to a general protocol. Separated proteins were then transferred to membranes using Trans-Blot® Turbo™ Mini PVDF Transfer Packs (Bio-Rad, Hercules, CA, USA). The membranes were blocked with Blocking One reagent (Nacalai Tesque, Kyoto, Japan) for 30 min and probed with Cry j 4–specific antiserum (1:500 dilution). After washing for 30 min, the membranes were incubated with ECL™ anti-rabbit IgG horseradish peroxidase–linked $F(ab')_2$ fragment (1:2500 dilution; GE Healthcare, Little Chalfont, UK) for 30 min. Finally, proteins were visualized with SuperSignal West Dura extended-duration substrate (Thermo Fisher). Chemiluminescence signals were captured using an LAS-3000 imaging system (Fuji Photo Film, Tokyo, Japan).

Subjects

A total of 21 Japanese cedar pollinosis patients and healthy subjects were selected from among the employees of Taiho Pharmaceutical Co., Ltd., between 2015 and 2016. Written informed consent was obtained from each subject. Clinical symptoms of seasonal allergic rhinitis suffered every spring were confirmed in the patients. Plasma samples were collected and analyzed for Japanese cedar pollen– and Japanese cypress pollen–specific IgE using ImmunoCAP (at the clinical test service of SRL, Tokyo, Japan). For inhibition assays, plasma samples were incubated with indicated concentrations of Cha o 3 for 2 days at 4 °C and analyzed by ImmunoCAP. The statistical significance of differences between the control and Cha o 3–inhibition groups was evaluated using paired t-tests. All studies were approved by the Research Ethics Review Committee of Taiho Pharmaceutical Co., Ltd. (approval number: S15-004).

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