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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

ABSTRACT

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 cause 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 1⁷ 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 (NH₂-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'-TCAAGCTTAAGTCCGTGGAYGAGGCAAC-3') and reverse (5'-GTTGATGCAACAATTKYTTG-3'), (5'-TTAAACCTCGAGTGCAGCAACAATKYTTGTTGGT-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'-GCCTAACTGCAGCTGTCCAGAGGAGCTG-3', and 3'-RACE was performed with two primers: 5'-GATGGAAGTATGATGCCAGT-3' and 5'-AGTGGATGTGTTAGATGGG-3'. Finally, the complete cDNA sequence of Cry j 4 was confirmed by

nested PCR using the following primers: forward, 5'-GATTCATGR-CACGCACAG-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).

Lymphocyte stimulation test

Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll–Paque density gradient centrifugation from whole blood collected in spring, as described previously.¹⁵ PBMCs (2×10^5) were stimulated with 10 μ g/mL of Cry j 1, Cry j 2 (Hayashibara, Okayama, Japan), Cha o 3, or rCry j 4 for 72 h. PBMCs were also treated with PBS(–) as negative controls. Incorporation of [³H]-thymidine (PerkinElmer, Waltham, MA, USA) was assessed according to a standard method as described previously.¹⁵ A total of 3 of 15 whole blood samples were from Japanese cedar ImmunoCAP–negative subjects (D, H and J, Table 1). Stimulation index (SI) = (mean cpm in triplicate cultures stimulated with antigen)/(mean cpm in negative control triplicate cultures).

Generation of Cha o 3–specific T-cell lines (TCLs) and T-cell clones (TCCs)

Cha o 3–specific TCLs were generated from PBMCs of Japanese cedar pollinosis patients as reported by Sone et al.¹⁰ Briefly, PBMCs (4×10^6) were stimulated with 10 μ g/mL of Cha o 3 in a 24-well flat-bottomed plate for 7 days. For these experiments, autologous complete medium (RPMI-1640 supplemented with heat-inactivated 10% autologous plasma and penicillin-streptomycin mixed solution [Nacalai Tesque]) was used. After culture for 7 days, 1 mL of supernatant was removed from each well, and then 1 mL of fresh complete medium containing 40 U/mL (final concentration: 20 U/mL) of recombinant human interleukin (IL)-2 (PeproTech, Rocky Hill, NJ, USA) was added for medium exchange. Medium exchange was performed every day for 9 days. When aggregation of proliferating T-cells was observed, the cultures were expanded using complete medium containing 20 U/mL of human IL-2. After an additional 9 days of culture, 4×10^5 cells of the Cha o 3–specific TCL were seeded with irradiated autologous PBMCs (1×10^5) for presentation of antigen (10 μ g/mL).

TCCs were generated from Cha o 3–specific TCL cells by limiting dilution (3 cells/well) and expanded by repeated Cha o 3 stimulation (10 μ g/mL) with 100 U/mL of recombinant human IL-2, 50 μ g/mL of recombinant human IL-4 (PeproTech), and irradiated autologous PBMCs once per week. The frequency of wells growing T cells

at the limiting dilution was 0.83% (Fig. 3E) and 1.33% (Fig. 3F), respectively. Proliferation of TCL cells and TCCs was assessed by monitoring [³H]-thymidine incorporation as described above. Statistical significance versus the PBS(–) treated condition was evaluated using Student's t-test.

Quantification of allergen-specific cytokine production

TCL cells (8×10^5) were stimulated with 10 μ g/mL of Cha o 3 or rCry j 4. After 72 h, the culture supernatant was collected and stored at -80°C until cytokine measurement. The levels of IL-5 and Interferon (IFN)- γ in the supernatant were determined using an ELISA kit (Affymetrix, Santa Clara, CA, USA). The statistical significance of differences between rCry j 4 treatment and PBS(–) (vehicle) treatment was evaluated using the Student's t-test.

Basophil activation test (BAT)

Heparinized whole blood was analyzed using an Allergenicity kit (Beckman Coulter, Indianapolis, IN, USA) according to the manufacturer's instructions. Briefly, indicated concentrations of allergen solutions and 20 μ L of cocktail composed of anti-human CD3-phycoerythrin (PE) cyanin 7 (PC7), anti-human CRTH2–fluorescein isothiocyanate (FITC), and anti-human CD203c-PE antibodies were added to 100 μ L of heparinized whole blood. The samples were immediately incubated at 37°C in a water bath for 15 min under shaded light conditions, after which 100 μ L of stop solution and 2 mL of “Fix-and-Lyse mixture” were added to each sample and incubated at room temperature for 10 min. Cells were collected by centrifugation and analyzed by flow cytometry (BD FACSCanto, BD Biosciences). A total of 3 of 20 whole-blood samples were from Japanese cedar ImmunoCAP–negative subjects (D, H and J, Table 1)

Results

Identification and cloning of the gene encoding Cry j 4

We initially tried to isolate the Cha o 3 homologous protein using a purification protocol similar to that used for Cha o 3 because a high degree of sequence identity was suggested by previous data.

Table 1

Levels of allergen-specific IgE in subject serum. Japanese cedar– and Japanese cypress–specific ImmunoCAP scores were measured in the spring of both 2015 and 2016.

Subject	2015 Japanese cedar		2016 Japanese cedar		2015 Japanese cypress		2016 Japanese cypress		Any allergic symptoms during cedar/cypress season
	score	UA/mL	score	UA/mL	score	UA/mL	score	UA/mL	
A	2	2.81	NT	NT	0	0.16	NT	NT	Yes
B	NT	NT	3	4.26	NT	NT	1	0.59	Yes
C	3	7.92	NT	NT	2	0.7	NT	NT	Yes
D	0	0.12	0	0.1	0	<0.1	0	0.1	No
E	4	29	4	35.3	2	1.53	2	2.04	Yes
F	3	14.9	4	18.6	2	2.56	3	3.51	Yes
G	NT	NT	3	13	NT	NT	2	1.85	Yes
H	0	<0.1	0	<0.1	0	<0.1	0	<0.1	No
I	NT	NT	4	28.1	NT	NT	2	1.27	Yes
J	0	<0.1	0	<0.1	0	<0.1	0	<0.1	No
K	4	26.3	4	33.4	2	1.33	2	1.5	Yes
L	2	3.05	3	5.28	1	0.49	1	0.47	Yes
M	3	8.14	NT	NT	1	0.53	NT	NT	Yes
N	5	55.4	NT	NT	3	5.72	NT	NT	Yes
O	3	11.5	4	20.4	1	0.61	1	0.42	Yes
P	3	7.53	3	9.11	2	1.22	2	1.11	Yes
Q	3	4.42	NT	NT	2	0.84	NT	NT	Yes
R	3	16	3	13.5	0	0.25	0	0.26	Yes
S	2	2.67	3	13.5	0	0.34	2	1.1	Yes
T	NT	NT	3	7.61	NT	NT	1	0.58	Yes
U	NT	NT	4	24.7	NT	NT	2	3.35	No

No, Subject had no allergic symptoms during Japanese cedar and Japanese cypress dispersion season. NT, Not tested.

An identical peptide corresponding to Cha o 3 (NH₂-WIVDEATGLR-COOH) was detected by LC-MS/MS analysis (Medical ProteoScope, Kanagawa, Japan) of the digest of the approximately 63 kDa SDS-PAGE band (data not shown). Therefore, we were convinced of the presence of a Cha o 3 homolog (i.e., Cry j 4) exhibiting a high degree of sequence identity. Applying this information as described in the Methods section above, we successfully cloned the gene encoding Cry j 4. The Cry j 4 cDNA coding region was 1659 base pairs in length. The complete deduced amino acid sequence comprised 552 amino acids, with a calculated molecular weight of 61,271 Da (Supplementary Fig. 1). Signal peptide prediction using GENETYX software (ver. 12.0.4) suggested that the peptide spanning from Met1 to Cys26 likely represents a signal peptide. Interestingly, the amino acid sequence of full-length Cry j 4 showed 85% identity and 97% similarity with that of Cha o 3 (Fig. 1). However, there was no identity between Cry j 4 and known Japanese cedar allergens such as Cry j 1, Cry j 2, Cry j 3,¹⁷ CJP-4,¹⁸ or CJP-6.¹⁹ A Pfam-A search (<http://pfam.xfam.org/search>) indicated that Cry j 4 contains a cellulase (glycosyl hydrolase family 5) motif from Pro68 to Gly351 (data not shown). The amino acid sequence of the cellulase motif of Cry j 4 was also similar (96% similarity) to that of Cha o 3, indicating that Cry j 4 functions as a cellulase.

The amount of Cry j 4 protein is low in Japanese cedar pollen

Although we tried to isolate Cry j 4 from Japanese cedar pollen by optimizing the isolation conditions described above, we could not obtain a sufficient amount for use in the various experiments. Therefore, rCry j 4 and anti-rCry j 4 rabbit antiserum were prepared as described in the Methods section above, and western blotting was used to compare the level of Cry j 4 protein in Japanese cedar pollen extract with that in Japanese cypress pollen extract. As shown in [Figure 2A](#), purified Cha o 3 was clearly detected using anti-rCry j 4 rabbit antiserum, suggesting that this antibody recognizes both Cry j 4 and Cha o 3. In addition, these data confirm the cDNA cloning results that indicate Cry j 4 is a homolog of Cha o 3. However, a relatively low level of Cry j 4 was detected in Japanese cedar pollen extract, despite the high level of Cha o 3 observed in Japanese cypress pollen extract ([Fig. 2A](#)). Similar results were observed for each pollen lysate ([Fig. 2B](#)). These data indicate that Cry j 4 expression in Japanese cedar pollen is quite low compared with Cha o 3 in Japanese cypress pollen extract. Therefore, we decided to use rCry j 4 for subsequent experiments to characterize its allergenicity.

Query	Subject	Score	Accession	Length	Identical	Similar
Query	1	60	MTSAGVAPTALRLTLALLLLVAAPSHSLPLLTRGRWIVDEATGLRVKLACVNWVGHPLEP	60	60	60
Cry j 4	Sbjct	1	MTATAMTTAALRLLLIA--LLLVAAPAECLPLRARGRWIVDEATGLRVKLACVNWPGHPLEP	58	58	58
Query	61	120	GLPEGLNRLPVATVAHTISSLGFNCRVRLTYSIHMLTRTSYTNATVAQTFARLNLTAAASG	120	120	120
Sbjct	59	118	GLPEGLNRLPVTTIAHTISSLGFNCRVRLTYSIQMVTEKSYTEATVGQTFALQNLTEPASG	118	118	118
Query	121	180	IEHNNPELLDLGHVAAAYHHVVAALSEAGVMVILDNHVSKPKWCCAVDDGNGFFGDRYFNP	180	180	180
Sbjct	119	178	IEGNPNPGLQLGHVAAAYDSIVAALAEAGVMVILDNHVSKPKWCCAVDDGNGFFGDSYFDP	178	178	178
Query	181	240	NTWVEGLGLMATYFNNTPNVVAMSLRNELRGNRSTPISWSRHMQWGAATVHKANPKVLVI	240	240	240
Sbjct	179	238	RLWQRLGLGLMATHFNNTPNVVAMSLRNELRGNRSTSARWSKYMQRGAATVHEANPNVLVV	238	238	238
Query	241	300	LSGLQFDTDLNFLPVLPTLPFKEKIVYEGHWYSFGVPWRTGLPNDVCKNETGRFKSNVG	300	300	300
Sbjct	239	298	LSGLHFDITLSFLPVLPTLPFKEKIVYEGHWYSFGVPWHDGLPNDICLNETSRFKDNIG	298	298	298
Query	301	360	FVTSSANATAAPLFMSEFGIDQRYVNDNDNRYLNCILAYLAEEDLDWALWTMGGSYYYRS	360	360	360
Sbjct	299	358	FLTSSINGTAAPLFVSEFGIDQRYVNDNDNRYLNCILAFLEEDVDWALWTMGGSYNYRS	358	358	358
Query	361	420	DKQPVKDFEETYGFFNHDWSRIRNPDIISRLKEIQQPIQDPYLPAGPYYQIIYHPASGLC	420	420	420
Sbjct	359	418	DKEPVQDFEETYGFFNRDWSRIRNPDIISRLKEIQQPIQDPYLPSPGPYYQIIYHPASGLC	418	418	418
Query	421	480	VESGIGNTVHLGSCQSVRSRWNYDASVKGPIGLMGSSSCISTQGNGLPAIMTENCAPNN	480	480	480
Sbjct	419	478	VESSIGNTIHLGSCQSVRSRWNYDASVEGPIGLMGSSSCISTQGNGLPAIMTEKCSAPNN	478	478	478
Query	481	540	TLWSTVSSAQLQLGTRVLGKDGKEKWMCLDGSKSPLISTNECICITDSHCYPKLNPEKQW	540	540	540
Sbjct	479	538	TLWSTVSSGQLQLGTRVFEDEGKEKWMCLDGSRSPITTECICITDSHCYPNQNPKEKQW	538	538	538
Query	541	554	FKVITTNKQLLHQL	554	471	471 / 554 (85%)
Sbjct	539	552	FKVITTNKQLLHQL	552	538	538 / 554 (97%)

Fig. 1. Analysis of the homology between Cry j 4 and Cha o 3. Amino acid sequences were aligned using GENETYX, ver. 12.0.4. Numbers indicate amino acid positions. Corresponding amino acid residues are linked by vertical lines.

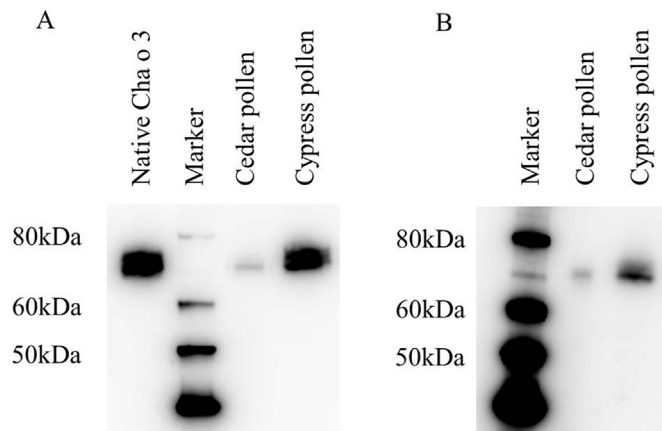


Fig. 2. Western blotting analysis of pollen extracts. Equal amounts of protein (52 µg/lane) from Japanese cedar or Japanese cypress pollen extract were separated by SDS-PAGE and detected using rCry j 4-specific antiserum. Cha o 3 was analyzed as a positive control. Pollen extract (A), pollen lysate (B).

Cha o 3-specific T cells exhibit cross-reactivity to Cry j 4

Lymphocyte stimulation tests were performed to determine whether Japanese cedar pollinosis patients are sensitized to Cry j 4. Table 1 summarizes the characteristics of the 21 subjects examined. PBMCs isolated from 15 subjects showed a proliferative response to rCry j 4 of a magnitude similar to that of the other cedar allergens and Cha o 3 (Fig. 3A). PBMCs from subject D, who was Japanese cedar ImmunoCAP-negative, showed a strong proliferative response to all of the allergens. PBMCs from subjects H and J

(ImmunoCAP-negative) were weakly stimulated by only rCry j 4 (4.08 and 4.22 in SI, respectively). To investigate the cross-reactivity between Cha o 3 and Cry j 4, we prepared Cha o 3-specific TCLs and evaluated their responses to Cry j 4 stimulation. Proliferative responses and cytokine production (especially of cytokines associated with Th2 responses) were observed for 2 of the 4 Cha o 3-specific TCLs (Fig. 3B, C, D). To further characterize the cross-reactivity, similar evaluations were performed using Cha o 3-specific TCCs. TCCs that reacted to Cha o 3 (Fig. 3F) or both rCry j 4 and Cha o 3 (Fig. 3E) were obtained, as expected. These data demonstrate that Cha o 3-specific T cells cross-react with Cry j 4.

Cha o 3-specific IgE cross-reacts with Cry j 4

To determine whether Cry j 4 evokes a type I allergic response in humans, a BAT was performed. Basophils from 7 of 20 (35%) subjects were BAT-positive to rCry j 4, and 6 of the 7 BAT-positive subjects were sensitized to Japanese cedar pollen (Fig. 4). Subject D also exhibited a positive BAT response. Therefore, we confirmed that Cry j 4 induces an IgE-dependent type I allergic response, particularly in Japanese cedar pollinosis patients. Basophils from 14 of 20 (70.0%) subjects, in particular, 13 of the 14 (92.9%) subjects who were Japanese cypress ImmunoCAP score >1 showed BAT-positive to Cha o 3.

Next, competitive ImmunoCAP analyses were performed using purified Cha o 3 to evaluate cross-reactivity at the IgE level. Briefly, indicated concentrations of Cha o 3 were added to the serum samples used for the ImmunoCAP tests. The results indicated that Japanese cypress-specific IgE binding (ImmunoCAP value) was inhibited in the serum from subject E. By contrast, Japanese cedar-specific IgE binding seemed slightly inhibited by Cha o 3

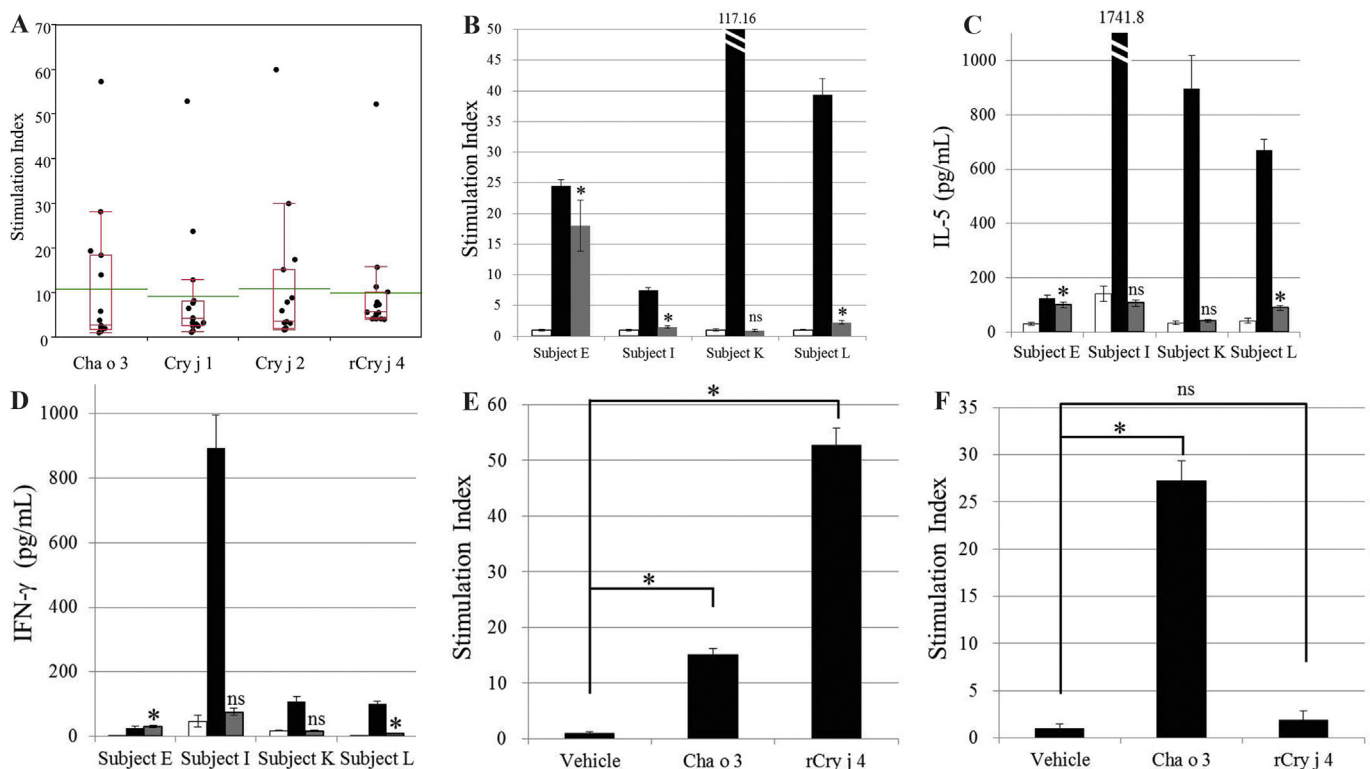


Fig. 3. Evaluation of Cry j 4-specific T-cell responses and associated cross-reactivity between Cry j 4 and Cha o 3. Proliferation analysis using the lymphocyte stimulation test. PBMCs from all subjects except A, C, M, N, O, and Q were used (A). Proliferation (B), IL-5 production (C), and IFN-γ production (D) of Cha o 3-specific TCLs in response to Cry j 4 or Cha o 3. White bars, PBS(-); black bars, Cha o 3; gray bars, rCry j 4 stimulation (B, C, D). Proliferation of Cha o 3-specific TCCs in response to Cry j 4 or Cha o 3 (E, F). Statistical significance versus the PBS(-) treated condition was evaluated using Student's t-test (**p* < 0.05). ns, not significant.

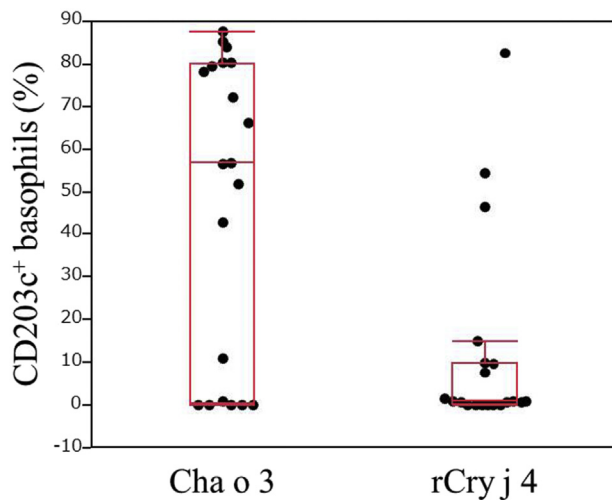


Fig. 4. Basophil activation test using rCry j 4 (3 µg/mL) and Cha o 3 (100 ng/mL). Whole blood samples from all subjects except subject T were analyzed.

(Fig. 5A). In addition to subject E, the same experiments were repeated using serum from three subjects (F, I and K, Table 1) sensitized to Japanese cedar and cypress pollen (Fig. 5B, C). The Japanese cypress-specific ImmunoCAP value was significantly

reduced (by 75.1%, Fig. 5B) by Cha o 3, confirming previous reports suggesting that Cha o 3 is a major allergen of Japanese cypress pollen. Interestingly, Cha o 3 competition partially reduced the Japanese cedar-specific ImmunoCAP value (by 17.7%, Fig. 5C). These data indicate that Cha o 3-specific IgE cross-reacts with an unknown protein (presumably Cry j 4) contained in the Japanese cedar pollen ImmunoCAP test.

Discussion

In the present study, we discovered Cry j 4 from Japanese cedar pollen and elucidated its immunologic characteristics. The results of cDNA cloning analyses indicated that the deduced amino acid sequence of Cry j 4 exhibits a high degree of identity and similarity (85% and 97%, respectively) to the major Japanese cypress pollen allergen Cha o 3. This high degree of identity suggests that Cry j 4 is also an allergen; however, we could not obtain native Cry j 4 protein in adequate amounts to confirm this due to its low content in Japanese cedar pollen. We had no choice but to evaluate its allergenicity using recombinant instead of native Cry j 4. rCry j 4 activated basophils from Japanese cedar pollinosis patients (Fig. 4). Subject D showed a strong proliferative response to all investigated Japanese cedar/cypress allergens and a positive BAT response to rCry j 4, despite being Japanese cedar ImmunoCAP-negative, suggesting that subject D was in a “positive sensitization and pre-disease” condition. PBMCs from Japanese cedar pollinosis patients

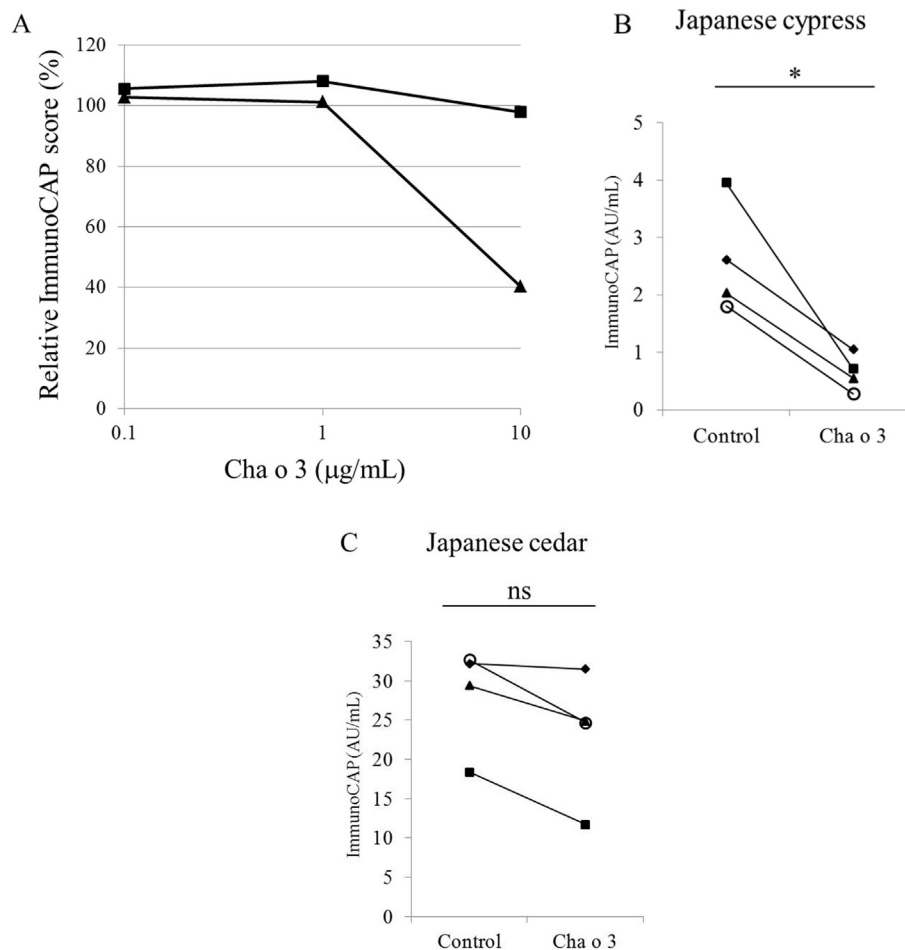


Fig. 5. ImmunoCAP inhibition assays using Cha o 3. Dose-dependent reductions in ImmunoCAP scores using serum from subject E. Squares, Japanese cedar-specific ImmunoCAP test; Triangles, Japanese cypress-specific ImmunoCAP test **(A)**. The results are shown as relative % value against the sample treated with vehicle instead of Cha o 3. Inhibition of Japanese cypress-specific ImmunoCAP **(B)** or Japanese cedar-specific ImmunoCAP **(C)** among four subjects (E, F, I and K). Diamonds: subject E, Squares: subject F, Triangles: subject I, Open circles: subject K. The competitive inhibition was performed with 10 µg/mL of Cha o 3. Statistical significance was evaluated using paired t-test (* $p < 0.05$). ns, not significant.

showed a proliferative response following rCry j 4 stimulation similar to that observed with other Japanese cedar allergens (Fig. 3A). In addition, Japanese cedar-specific ImmunoCAP scores were reduced, despite the addition of Cha o 3, which is a known allergen of Japanese cypress pollen (Fig. 5C). These data support the hypothesis that Cry j 4 is a counterpart to Cha o 3 in Japanese cedar pollen. We are therefore convinced that Cry j 4 plays a role in the onset of Japanese cedar/cypress pollinosis as a Japanese cedar pollen allergen. However, it should be taken into account that we could not directly evaluate binding of IgE to native form Cry j 4 due to the poor yield. Further investigations will be needed to accurately determine the rate of sensitization to Cry j 4 among Japanese cedar/cypress pollinosis patients and the contribution of native Cry j 4 to the induction of allergic symptoms in spring.

Next, we evaluated the homology between Cry j 4 and Cha o 3. The similarity between Cry j 4 and Cha o 3 (85% identity) was significant and comparable to the identity between the group 1 pollen allergens Cry j 1 and Cha o 1 (80%) and the group 2 pollen allergens Cry j 2 and Cha o 2 (74%). Indeed, it is reported that these group 1 and 2 allergens belong to same protein families, pectate lyase and polygalacturonase, respectively.^{20,21} Cry j 4 and Cha o 3, which belong to the cellulase family, would form a new group of plant pollen allergens among trees of the *Cupressaceae/Taxodiaceae* families, as with group 1 and group 2 pollen allergens.

Regarding the high degree of amino acid sequence similarity between Cry j 4 and Cha o 3, we found that Cry j 4 cross-reacts with Cha o 3 at both the T- and B-cell levels (Fig. 2, 3, 5). Although the considerable difference of BAT-positive rate was observed between rCry j 4 and Cha o 3 (Fig. 4), this was most likely due to the difference in conformation between the native forms of Cry j 4 and rCry j 4. Misfolded rCry j 4 might be contained and showed partial conformational IgE binding. Therefore, we also determined that an ImmunoCAP inhibition assay using native Cha o 3 would be more accurate for demonstrating cross-reactivity, for the same reason (Fig. 5). The results of our recent study indicated that Cha o 3 cross-reacts with an unknown component in Japanese cedar pollen standardized extract.¹⁵ Based on the results of the present study, there is little doubt that Cry j 4 is the previously unknown allergen in Japanese cedar pollen. Group 1 and group 2 pollen allergens of *Cupressaceae* trees are known to share allergenic components and cross-react among pollinosis patients. For example, Cry j 1, Cha o 1, and Jun a 1 cross-react with each other at the T-cell and IgE-binding levels. The cross-reactivity between Cry j 4 and Cha o 3 observed in the present study support the results of these previous studies.

Owing to their cross-reactivity, we could not determine whether partial inhibition of IgE binding to Japanese cedar-specific ImmunoCAP by Cha o 3 protein was Cha o 3-specific IgE or Cry j 4-specific IgE (Fig. 5C). However, the difference in Cry j 4 and Cha o 3 content in the pollen suggests an answer. We speculate that Cha o 3-specific IgE is induced initially and reacts with Cry j 4 in the next Japanese cedar pollen season, based on the observation that Cha o 3 expression was obviously high compared with that of Cry j 4 in Japanese cedar pollen. Similarly, it is likely that Cha o 3-induced specific Th2 cells are activated by Cry j 4 in early spring, which is Japanese cedar pollen season, before Japanese cypress pollen dispersion season. On the other hand, there was no significant difference between the cedar-sensitized group and group sensitized with both cedar and cypress allergens with regard to rCry j 4 specific T cell proliferation (data not shown). Further experiments focused on the cytokine profile in lymphocyte stimulation test supernatants might be useful to obtain some hints for discussion regarding the order of sensitization to Cry j 4 and Cha o 3 because the lymphocyte stimulation test would detect both Th1 and Th2 responses. In terms of T-cell cross-reactivity between Cry j 4 and Cha o 3, two patterns of TCCs activated by one or both allergens

were observed (Fig. 3). These findings suggest that there are both corresponding T-cell epitopes and different epitopes in pollinosis patients. It would be difficult to determine which allergen is the cause of the original sensitization due to the shared T-helper epitopes in Cry j 4 and Cha o 3. Major T-cell epitopes of Cry j 4 and Cha o 3 in humans should be elucidated to promote better understanding of sensitization to Cry j 4 and/or Cha o 3. Based on these findings, it is our opinion that the pathogenic roles of both Cry j 4 and Cha o 3 should be taken into account during the Japanese cedar and cypress dispersion seasons in order to obtain a better understanding of Japanese cedar/cypress pollinosis. In addition, further exploratory studies, particularly in *Cupressaceae/Taxodiaceae* family trees located around the world, are needed in order to clarify which disorders are attributable to cellulase-family allergens.

We must also consider Cry j 4 from the perspective of ASIT against Japanese cedar pollinosis. As described in the Introduction section, allergic symptoms and QOL may worsen despite Japanese cedar-specific ASIT, especially during Japanese cypress season.¹³ We identified Cha o 3 to investigate the cause of the worsening of allergic symptoms despite ASIT, and in fact, ASIT using Japanese cedar extract could not completely control Cha o 3-specific Th2 responses, despite the fact that Cry j 1-, Cry j 2-, and Cha o 1-associated Th2 cytokine production was completely abrogated.¹⁵ Based on these results, we hypothesized that either the amount of the Cha o 3 homolog in Japanese cedar pollen extract is not sufficient for desensitization or its amino acid sequence identity is low. It is evident from the results of the present study that lack of Cha o 3-specific Th2 inhibition would be determined by the content of Cry j 4 in Japanese cedar pollen extract rather than low identity of amino acid sequence (Fig. 2A, B). In fact, no signal was detected by western blotting analysis, although we tried to confirm the presence of Cry j 4 in standardized Japanese cedar pollen extract (data not shown). Therefore, ASIT using Japanese cedar pollen extract would be an insufficient approach for controlling allergic symptoms during Japanese cedar/cypress season if one of the major causes of the therapeutic effect attenuation is remaining Cha o 3-specific Th2 responses. Moreover, because approved ASIT drugs for Japanese cedar pollinosis in Japan are only standardized Japanese cedar pollen extract for subcutaneous injection or its sublingual route formulation,²² adequate Th2 control of Cha o 3 cannot be achieved under the current standard treatment. Development of an ASIT using standardized Japanese cypress pollen extract which contains considerable Cha o 3 or purified Cha o 3 with the standardized Japanese cedar pollen extract might improve the therapeutic effect against both Japanese cedar and Japanese cypress pollinosis through the inhibition of group 1-, group 2-, and Cry j 4-Cha o 3-specific Th2 responses.

In conclusion, a novel Japanese cedar pollen allergen, Cry j 4, was identified from Japanese cedar pollen and found to be a homolog of the major Japanese cypress pollen allergen, Cha o 3. Although Cry j 4 alone would not be a major allergen in Japanese cedar pollen due to its low content, Cry j 4 could play an essential pathogenic role in the geographic conditions unique to spring in Japan, which is characterized by heavy release of Japanese cypress pollen after the release of Japanese cedar pollen. The discovery of Cry j 4 enhances our understanding of Japanese cedar/cypress pollinosis. In addition, Cry j 4 revealed a limitation in the efficacy of ASIT in that it targets only Japanese cedar extract in Japanese cedar/cypress pollinosis patients. 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. We expect the results of the present study will lead to improvements in the ASIT approach for treating Japanese cedar/cypress pollinosis, which afflicts millions of people in Japan.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.alit.2018.02.004>.

Conflict of interest

TO, YT, and ES are employees of Taiho Pharmaceutical Co., Ltd. and TU is a board member of Taiho. AY has no conflict of interest.

Authors' contributions

TO designed all studies, contributed to data collection and analysis, and wrote the manuscript. YT contributed to data collection and analysis. AY and ES contributed to analysis and interpretation of data, and assisted in the preparation of the manuscript. TU contributed to the conception of this work and gave final approval of the manuscript. All authors critically reviewed and approved the final manuscript.

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10	20	30	40	50	60	610	620	630	640	650	660	1210	1220	1230	1240	1250	1260
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70	80	90	100	110	120	670	680	690	700	710	720	1270	1280	1290	1300	1310	1320
GCGGCGCGGACAGTGCCTGCCGCTACGGGCACGTGGCAGATGGATCGTGGACGAGGCA	ATGCAGCGGGGTGCGGCTACCGTCCACGAGGCCAACCCAAATGTCCTCGTTGCTCTCG	TCCAGCATTGGAAACACCATTCTAGGATCATGCCAGAGTGTGAGAAGCAGATGGAAC															
A A P A E C L P L R A R G R W I V D E A	M Q R G A A T V H E A N P N V L V V L S	S S I G N T I H L G S C Q S V R S R W N															
130	140	150	160	170	180	730	740	750	760	770	780	1330	1340	1350	1360	1370	1380
ACGGGGCTGAGAGTAAAGTAGCATGCGTCAATTGGCCGGGCCACCTGGAGCCCGGGTTA	GGGCTACACTTCGACACCATCCTCAGCTTCTTACCGGTCTACCACTTACTTTACCTTTT	TATGATGCCAGTGTGGAAGGCCCAATTGGACTAATGGGAAGTTCATCCTGCATTCCACT															
T G L R V K L A C V N W P G H L E P G L	G L H F D T I L S F L P V L P V T L P F	Y D A S V E G P I G L M G S S S C I S T															
190	200	210	220	230	240	790	800	810	820	830	840	1390	1400	1410	1420	1430	1440
CCGGAAGGTCTCAACCGGTTGCCGGTTACCAATAGCGCACACCATCAGCTCTCTGGGC	AAAGAGAAAATTGTGTATGAAGGGCACTGGTACTCCTTCGGTGTGCCTGGCATGATGGC	CAAGGAAATGGGTGCGCTGCAATTATGACAGAAAAATGTTCTGCCCCCAACACACTCTG															
P E G L N R L P V T T I A H T I S S L G	K E K I V Y E G H W Y S F G V P W H D G	Q G N G L P A I M T E K C S A P N N T L															
250	260	270	280	290	300	850	860	870	880	890	900	1450	1460	1470	1480	1490	1500
TTCAATTGTGTCGCGCTCACTACTCCATTAGATGGTCACGGAGAAGAGCTACACAGAG	TTGCCTAATGACATTTGTTTGAATGAGACGTGCGGGTTTAAGGATAATATTGGGTTTTG	TGGAGCACAGTCTCCTCTGGACAGTGCAGTTGGGCACTAGAGTTTTTATGAGGATGGG															
F N C V R L T Y S I Q M V T E K S Y T E	L P N D I C L N E T S R F K D N I G F L	W S T V S S G Q L Q L G T R V F D E D G															
310	320	330	340	350	360	910	920	930	940	950	960	1510	1520	1530	1540	1550	1560
GCCACGGTTGGGCAGACTTTTGGCAGCTAAATCTGACAGAACCCGCTCGGGAATCGAG	ACCTCGTCGATTAATGGTACTGCGGCACCACTTTTGTAGCGAGTTCGGAATTGATCAG	AAAGAGAAGTGGATGTGTTTAGATGGGAGTAGAAGTCCATTGATTACAACAACATGAATGC															
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370	380	390	400	410	420	970	980	990	1000	1010	1020	1570	1580	1590	1600	1610	1620
GGCAACAATCCGGGGTTCCTCCAATTGGGCCACGTGGCTGCATACGATAGCATCGTGGCG	AGATACGTGAATGATAATGATAACAGGTATTTGAACTGTATCTTGGCTTTTCTGGCGGAG	ATCTGCATCACTGACTCTCACTGCTATCCAAATCAGAATCCAGAAAAGCAGTGGTTAAA															
G N N P G F L Q L G H V A A Y D S I V A	R Y V N D N D N R Y L N C I L A F L A E	I C I T D S H C Y P N Q N P E K Q W F K															
430	440	450	460	470	480	1030	1040	1050	1060	1070	1080	1630	1640	1650			
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A L A E A G V M V I L D N H V S K P K W	E D V D W A L W T M G G S Y N Y R S D K	V I T T N K Q L L H Q L *															
490	500	510	520	530	540	1090	1100	1110	1120	1130	1140						
TGCTGTCTGTGGATGATGGCAATGGGTTTTTCGGTGACAGTTACTTCGACCCTAGATTG	GAGCCCGTTACGAGCTTTGAGGAGACCTACGGCTTTTCAATCGTGACTGGAGTCGCATC																
C C A V D D G N G F F G D S Y F D P R L	E P V Q D F E E T Y G F F N R D W S R I																
550	560	570	580	590	600	1150	1160	1170	1180	1190	1200						
TGGCAACGAGGGCTAGGTCTAATGGCTACACATTTCAATAACACGCCCAACGTCGTCGCC	AGGAATCCTGACTTTATTCTCGCCTTAAGGAAATTCACAGCCCATTCAGACCCCTTAC																
W Q R G L G L M A T H F N N T P N V V A	R N P D F I S R L K E I Q Q P I Q D P Y																

Supplementary Fig. 1. Cloned cDNA sequence of the gene encoding Cry j 4 and deduced amino acid sequence of the protein product.

The nucleotide sequence reported was submitted to the GenBank/NCBI Databases under accession number LC214561.