

Molecular cloning and characterization of a new Japanese cedar pollen allergen homologous to plant isoflavone reductase family

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Summary

Background Japanese cedar (*Cryptomeria japonica*) pollen is a major cause of seasonal pollinosis, and more than 10% of Japanese people suffer from this allergic disorder. However, only two major pollen allergens, Cry j 1 and Cry j 2, have been identified and exclusively characterized.

Objective The aim of this study was to explore and identify important Japanese cedar pollen allergens other than Cry j 1 or Cry j 2.

Methods *C. japonica* cDNA library was immunoscreened by rabbit antiserum raised against a partially purified cedar pollen allergen fraction. An isolated cDNA clone was inserted into a glutathione S-transferase (GST)-tagged *Escherichia coli* expression vector to obtain recombinant GST fusion protein. Non-fusion recombinant protein was purified by glutathione Sepharose affinity chromatography in conjunction with factor Xa cleavage of the GST moiety. IgE-binding ability of the recombinant protein was then evaluated by western blot analysis and enzyme-linked immunosorbent assay (ELISA).

Results The cDNA encodes 306 amino acids with significant sequence similarity to those of plant isoflavone reductase-like proteins, which include a recently identified birch pollen allergen Bet v 5. Western blot analysis demonstrated that recombinant protein was recognized by cedar pollinosis patient IgE. In contrast to Bet v 5 being reported as a minor allergen, the recombinant protein exhibited 76% IgE binding frequency (19/25) against pollinosis patients.

Conclusion Here we identified the third member of Japanese cedar pollen allergen homologous to isoflavone reductase. Its high IgE-binding frequency implicates that the isoflavone reductase homologue might be an additional major pollen allergen in *C. japonica*.

Keywords allergen, isoflavone reductase, Japanese cedar pollen, pollinosis

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Introduction

Pollinosis is a serious allergic disease world-wide. In Japan, Japanese cedar (*C. japonica*) pollen is a major spring aeroallergen [1], and elicits pathogenic symptoms including rhinitis and/or conjunctival congestion accompanied by pollen-specific IgE production. In addition to *C. japonica* pollen, Japanese cypress (*Chamaecyparis obtusa*) pollen represents an alternative aeroallergen, as these two species show IgE cross-reactivity [2]. To establish effective diagnosis and specific immunotherapy for cedar pollinosis, identification and immunochemical characterization of important allergen molecules is a crucial step.

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Previous studies identified two major Japanese cedar pollen allergens designated as Cry j 1 [1] and Cry j 2 [3]. cDNA cloning has revealed that *cry j 1* cDNA encodes a polypeptide showing homology to the major short ragweed allergen Amb a 1 family [4], whereas *cry j 2* gene codes for a polygalacturonase-like protein [5,6]. Taniguchi et al. have shown that Cry j 1 has pectate lyase enzyme activity [7]. Because of their high frequency of sensitization [8], intensive immunochemical analyses have been carried out on these two allergens including the identification of T cell determinants [9–11]. Based on the accumulated T cell epitope information, Sone et al. have engineered a recombinant Cry j 1/Cry j 2 epitope cassette for specific immunotherapy [12]. It has also been demonstrated by animal study that oral administration of a dominant T cell epitope peptide inhibits allergen-specific T cell response in Cry j 2-primed mice [13], implicating a possible oral immunotherapy of Japanese cedar pollinosis. Recently, a Japanese cypress counterpart of Cry j 1 or Cry j 2, designated as Cha o 1 or Cha o 2, has also been identified [14,15]. Although immunochemical information was thus exclusively accumulated on these two allergen

members, comprehensive characterization of other important Japanese cedar pollen allergens has yet to be carried out. In the present study, we report the identification of the third member of Japanese cedar pollen allergen structurally belonging to the plant isoflavone reductase family.

Materials and methods

Japanese cedar pollen, reagents and pollinosis patient sera

Pollen was collected from *C. japonica* trees in Hiroshima Prefecture, and stored at -80°C until use for antigen preparation or cDNA library construction. General chemicals used in this study were from Katayama Chemical (Osaka, Japan) unless specifically indicated. Sera were collected from Japanese cedar pollinosis patients whose radioallergosorbent test (RAST) scores were over 3. Control non-allergic sera were collected from healthy volunteers at Hiroshima University.

Preparation of Japanese cedar pollen extract

Pollen (80 g) was stirred in 3.2 L of phosphate-buffered saline (PBS) at 4°C for 4 h, and homogenate was centrifuged at 6800 g for 30 min. The supernatant was applied to ammonium sulphate precipitation at 80% saturation. Resulting precipitate was then dissolved in distilled water (DW) and dialysed overnight against DW. After centrifugation at 6500 g for 30 min, supernatant was filtered via a 0.22 μm filter unit (Millipore, Bedford, MA, USA) to obtain crude Japanese cedar pollen extract. The antigen extract was freeze-dried and stored at -80°C until use.

Preparation of antiserum against 30 kDa Japanese cedar pollen antigen fraction

Crude *C. japonica* extract was dialysed against 20 mM sodium acetate buffer (pH 5.0), and then applied onto HiLoad 26/10 SP Sepharose HP column (Amersham Pharmacia Biotech, Uppsala, Sweden). Adsorbed fraction was obtained by eluting with 1 M NaCl/20 mM sodium acetate buffer, and 30 kDa antigen fraction was partially purified by preparative sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the AE-6400 system (Nippon Eido, Tokyo, Japan) according to the manufacturer's instruction. To obtain antiserum against the 30 kDa antigen fraction, Japanese white rabbits (Charles River Japan) were immunized intramuscularly with 100 μg of the antigen that was pre-adsorbed with Freund's complete adjuvant (Wako Pure Chemical, Osaka, Japan). Immunization was carried out five times every 2 weeks. This animal experiment was carried out in accordance with the Guide for Animal Experimentation, Hiroshima University, and the Committee of Research Facilities for Laboratory Animal Science, Hiroshima University School of Medicine. Specificity of antiserum was confirmed by western blot analysis as described below. To test whether antiserum-reactive protein also binds pollinosis patient IgE, natural 30 kDa cedar pollen antigen was purified via affinity column chromatography using specific antibody-immobilized Formyl-Cellulofine (Seikagaku Kogyo, Tokyo Japan), and then subjected to western blot analysis.

Construction of a C. japonica pollen cDNA library

To extract total RNA, pollen (8 g) was homogenized in an extraction buffer [mixture of 10 mL of 100 mM NaCl, 10 mM EDTA, 1% SDS, 100 mM Tris-HCl (pH 8.0), 2.5 mL

β -mercaptoethanol, 10 mL phenol:chloroform:isoamylalcohol (25:24:1, v/v/v)], and stirred at room temperature (25°C) for 40 min. After centrifugation at 5900 g for 20 min, supernatant was applied to phenol extraction (three times), followed by chloroform:isoamylalcohol (24:1, v/v) extraction for phenol depletion. RNA was then obtained via successive isopropanol precipitation and LiCl precipitation. poly (A)⁺ RNA was selected with Oligotex dT30 super (Takara, Kyoto, Japan). Double-stranded cDNA was synthesized by using TimeSaver cDNA synthesis Kit (Amersham Pharmacia Biotech). *C. japonica* cDNA library was constructed on λ ZIPLOX (Life Technologies, Frederick, MD, USA) by inserting the cDNA into *NotI/SalI* site of the vector arm.

Immunoscreening of cDNA library and DNA sequencing

C. japonica cDNA library (5×10^4 pfu) was screened with rabbit antiserum raised against the 30 kDa *C. japonica* pollen antigen fraction (see above), and isolated positive cDNA clone (CJP-6) was recovered as a phagemid (pZL1-CJP6) via *Cre/loxP* system-mediated *in vivo* excision in *E. coli* DH12S strain. Both strands of the insert cDNA were sequenced using the ABI PRISM 310 Genetic Analyser (PE Biosystems, Norwalk, CT, USA).

Expression and purification of recombinant protein

CJP-6 cDNA fragment (nucleotide no. 94–1014, corresponding to amino acid no. 1–306, see Fig. 2) was prepared by polymerase chain reaction (PCR). The PCR product was subcloned into pGEM-T easy vector (Promega, Madison, WI, USA), and *EcoRI*-cleaved CJP-6 cDNA fragment was then cloned into the GST-tagged pGEX-3X vector (Amersham Pharmacia Biotech). *E. coli* BL21 strain was transformed with the expression plasmid, and transformant was cultured in $2 \times \text{YT}$ medium (2 L) supplemented with 0.1% isopropyl-1-thio- β -D-galactoside (IPTG) to express recombinant CJP-6 as a GST-fusion protein. Recombinant protein was purified by affinity chromatography using glutathione Sepharose 4B column (Amersham Pharmacia Biotech) according to the manufacturer's instruction. Briefly, harvested bacterial cells were lysed under sonication in an extraction buffer (3 mM EDTA, 1% Triton X-100, PBS). After centrifugation, the supernatant was mixed with 2 mL glutathione Sepharose 4B and incubated overnight at 4°C with gentle rotation. After washing the column with the extraction buffer, immobilized recombinant protein was cleaved by digestion with Factor Xa (Amersham Pharmacia Biotech).

SDS-PAGE and western blot analyses

Protein samples (crude pollen extract, 30 kDa antigen fraction, or recombinant protein) were resolved by SDS-PAGE according to the discontinuous buffer system by Laemmli [16] on a 12.5% acrylamide slab gel. Fractionated proteins were then visualized by 0.25% Coomassie brilliant blue (CBB) staining. For western blotting, fractionated protein was electronically transferred onto a polyvinylidene difluoride filter (Immobilon P, Millipore), and probed with primary antibody (*C. japonica*-allergic patient IgE or rabbit anti-30 kDa pollen antigen IgG). To detect human IgE binding, blots were stained with secondary biotin-conjugated goat anti-human IgE antibody (Zymed, San Francisco, CA, USA), followed by reaction with horseradish peroxidase (HRP)-conjugated streptavidin

(Zymed). In the case of 30 kDa antigen detection via specific antibody, blots were incubated with HRP-conjugated goat anti-rabbit IgG antibody (New England Biolabs, Beverly, MA, USA). Protein bands were visualized by reaction with diaminobenzidine (DAB) substrate, or on X-ray film (Fuji Photo Film, Tokyo, Japan) using ECL Plus western blotting detection reagent (Amersham Pharmacia Biotech).

Enzyme-linked immunosorbent assay

IgE binding frequency of recombinant CJP-6 protein was evaluated by enzyme-linked immunosorbent assay (ELISA). For antigen coating, 50 µL of recombinant antigen solution (500 ng/mL, in 100 mM carbonate buffer, pH 9.3) were added to 96-well microtitre plates (Falcon 3912, Becton Dickinson Labware, Lincoln Park, NJ, USA), and incubated at room temperature for 2 h. After blocking the plate with 3% skim milk/PBS, 50 µL of diluted cedar pollinosis patients sera ($\times 40$ diluted with blocking buffer) were supplemented into the well and incubated overnight at 4 °C. Then 50 µL of secondary biotin-conjugated anti-human IgE ($\times 1000$ dilution, purchased from Zymed) were added and incubated at room temperature for 2 h, followed by addition of 50 µL of alkaline phosphatase-conjugated streptavidin ($\times 1000$ diluted, Zymed). For enzyme reaction, AttoPhos substrate solution (Boehringer Mannheim, Mannheim, Germany) was added and incubated at room temperature for 30 min. Then fluorescence intensity of each sample well was analysed using CytoFluor II microplate reader (PE Biosystems).

Competitive enzyme-linked immunosorbent assay

Purified recombinant CJP-6 (0.2 µg/mL in 100 mM carbonate buffer, pH 9.3) was coated onto a 96-well microtitre plate by incubation at room temperature for 2 h, and then blocked overnight at 4 °C with 1% bovine serum albumin, 5% skim milk and 0.1% Tween-20 in PBS. Prior to reaction with the coated antigen, cedar pollinosis patients sera (a pool made by five patients, $\times 40$

diluted by blocking buffer) were pre-incubated overnight at 4 °C with serial dilutions of competitors (*C. japonica* pollen extract, recombinant CJP-6, or irrelevant ovalbumin). The pre-adsorbed sera were then applied onto the microtitre plate for testing IgE cross-reactivity. Subsequent secondary antibody staining and signal detection was carried out as described above.

Results and discussion

cDNA cloning of an isoflavone reductase homologue in *C. japonica* pollen

Prior to comprehensive allergen characterization in *C. japonica* pollen, we performed western blot analysis of the pollen extract using Japanese cedar pollinosis patient IgE pool as a probe. We found more than 10 IgE-binding components other than Cry j 1 and Cry j 2 on the blot (Fig. 1a), suggesting the existence of new allergen molecules. Among these IgE-reactive protein bands, a smear signal around 30 kDa is relatively more intense than the others (Fig. 1a, arrowhead). Thus, we decided to focus initial allergen exploration on this 30 kDa molecular weight region.

We first semipurified the 30 kDa antigen fraction via successive SP Sepharose cation-exchange chromatography and preparative SDS-PAGE (Fig. 1b). Then we prepared rabbit antiserum against this antigen for cDNA cloning. Western analysis demonstrated that obtained antiserum showed positive IgG binding to the purified antigen (Fig. 1b, arrowhead). We next tested whether pollinosis patient IgE also recognized antiserum-reactive protein. Immunoreactive 30 kDa cedar pollen antigen was purified from crude *C. japonica* extract using a Formyl-Cellulofine affinity column on which specific antibody was covalently immobilized. Western blot analysis indicated that the affinity-purified 30 kDa antigen was recognized by a pollinosis patient IgE pool (Fig. 1c), again confirming that antiserum-reactive protein fraction contains IgE-binding component.

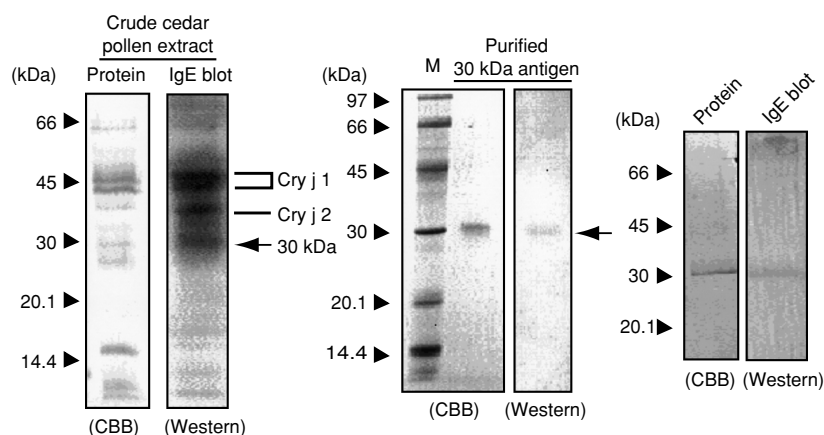


Fig. 1. (a) SDS-PAGE and IgE immunoblotting analyses of crude *C. japonica* pollen extract. Crude extract was run on 12.5% polyacrylamide gel, and then subjected to 0.25% CBB staining or western blot analysis with pollinosis patient IgE pool ($n = 20$, RAST score > 3). Positive IgE staining around 30 kDa was shown arrowhead. Major *C. japonica* allergens Cry j 1 and Cry j 2 were also indicated. (b) Purification of 30 kDa cedar pollen antigen fraction and preparation of rabbit antiserum. The 30 kDa antigen was fractionated via SP Sepharose cation-exchange chromatography and preparative SDS-PAGE. Purified 30 kDa fraction (CBB-stained, in left panel) was immunoblotted with rabbit antiserum (DAB staining, right panel). (c) Specific antiserum-reactive protein component is also recognized by *C. japonica*-allergic patient IgE. Natural 30 kDa antigen was purified from crude extract by affinity chromatography using rabbit specific antibody-immobilized Formyl-Cellulofine column, and was then subjected to western blot analysis with pollinosis patient IgE pool (DAB staining).

1		
CCACGCGTCCGGTTGATCTTTGTTAAAGTATCATCTTGCTACTTAGGAATTGAAATAGAT	60	
CAGAAATTAATCTTTATTCTCTTGAATACTGAAGATGGGAGGAAGTAGGTTTTGATAATA	120	
		M G G S R V L I I 9
GGAGGAAGTGGTTACATTGGTAGACATGTGACCAATGCAAGCCTTGCCCAAGGTCAACCC	180	
<u>G G T G Y I G</u> R H V T <u>N A S</u> L A Q G H P	29	
ACTTTCCTCCTTGTAGAGAAATCACACCTTCCAACCTGAAAAGGCCAGCTGCTAGAG	240	
T F L L V R E I T P S N P E K A Q L L E	49	
TCCTTCACATCCAAAGGAGCAACTCTGGTTCAAGGGTCCATAGATGATCATGCAAGCCTT	300	
S F T S K G A T L V Q G S I D D H A S L	69	
GTAGCAGCACTGAAGAAGTGGATGTAGTCATTTCAACATTGGGTGCACCACAGATAGCA	360	
V A A L K K V D V V I S T L G A P Q I A	89	
GATCAGTTCAATCTAATCAAGGCTATCAAAGAAGTTGGAACATTAAAGAGGTTTTTCCCA	420	
D Q F N L I K A I K E V G T I K R F F P	109	
TCAGAGTTTGGGAATGATGTTGATAAGCACCATGCAGTGGAGCCTATGAAGAGTATGTTT	480	
S E F G N D V D K H H A V E P M K S M F	129	
GACTTGAAAATCAAACCTTCGCCGACTATTGAGGCAGAAGGCATTCCACATACATATGTT	540	
D L K I K L R R T I E A E G I P H T Y V	149	
GTTCCACATTGTTTTGCAGGCTACTTTCCTACTAATCTTGACAGCTTGGGCTAGCTGCC	600	
V P H C F A G Y F L T N L A Q L G L A A	169	
CCGCCAAGGGATAAAATTTGTTATTTATGGAGATGGAACACCAAGCTGTATATATGAAG	660	
P P R D K I V I Y G D G T T K A V Y M K	189	
GAAGAAGATATTGGGACATTCACAATCAAGGCAGTGGATGATCCAAGGACTTTGAACAAG	720	
E E D I G T F T I K A V D D P R T L <u>N K</u>	209	
ACCCTCTACTTGAAGCCTCCTGCCAATACTATCTCCACCAATGATCTCGTAGCCCTTTGG	780	
<u>T</u> L Y L K P P A N T I S T N D L V A L W	229	
GAGGCGAAGATAGGGAAGACTTTGGAGAAGGTCTATCTATCTGAGGAGCAGGTTCTTAAA	840	
E A K I G K T L E K V Y L S E E Q V L K	249	
CTACTCCAAGATACACCATTTCAGGACTTTTATGGTATCCATTTTTCACACCATTATAT	900	
L L Q D T P F P G T F M V S I F H T I Y	269	
GTGAAAGGAGATCAAACCAATTTTCAGATTGGACCTGATGGGGTTGAGGCCAGTGCCTG	960	
V K G D Q T N F Q I G P D G V E A S A L	289	
TACCCAGATGTGAAATATACAACCTGTTGAAGAGTATATCAGTGCATTTGTATGAATCATA	1020	
Y P D V K Y T T V E E Y I S A F V *	306	
TGATGAGATAAAAGTTTATATGATTGAATAAGTTTTCCTAAGTGCTTGAAAAACCACTGA	1080	
ATGAGTTTGTGAAATGTACAGGACAAATCACTGATAAGTTTAGTATTGTAGAAGGGTAGA	1140	
TTTAGAAGCATGTCTTGACCTTCTCAAGTTGTAGTTGCATTTTCTGTGAGCAAAATAAT	1200	
TTTTAGTTTCATTTAAAAA	1233	

Fig. 2. Nucleotide sequence of CJP-6 cDNA and its deduced amino acid sequence. Putative NAD(P)H binding domain (amino acid no. 10–16) is open boxed, and two potential *N*-glycosylation sites (Nos 21–23, 208–210) are underlined. Putative polyadenylation signal (AATAAA, nucleotide no. 1994–99) is also underlined. Nucleotide and amino acid sequence information of the CJP-6 are available on the GenBank Sequence Database under accession number AY028631.

Immunoscreening of *C. japonica* cDNA library with the anti 30 kDa antigen serum yielded one positive clone, CJP-6. Sequencing analysis revealed that the CJP-6 cDNA consisted of 1233 nucleotides containing a 921-bp open reading frame (ORF, see Fig. 2). The ORF encodes a 306 amino acid polypeptide with a calculated molecular weight of 33 573. We find two potential *N*-glycosylation sites (Nos 21–23 and 208–210) on the ORF, although actual *N*-glycosylation of these sites remains to be demonstrated.

The BLAST amino acid similarity search [17] indicated that deduced CJP-6 amino acid sequence showed significant

homology with a family of isoflavone reductase (IFR) from plants. Figure 3 shows typical multiple sequence alignment of CJP-6 polypeptide with IFR family members. One structural feature of the IFR family is the conserved GX(X)GXXG NAD (P)H-binding domain [18]. In the CJP-6 sequence, we found this consensus sequence (amino acid no. 10–16, open boxes or asterisks in Fig. 2 or Fig. 3, respectively). Maximum sequence identity (73%) was obtained when compared with pine (*P. taeda*) phenylcoumaran benzylic reductase (GenBank accession No. AF242490 [19]). Significant matching was also noted with vegetable IFRs such as from soybean (62% identity,

CJP-6	1: M-G-G-SRVLITGGTGYIGRHVTNASTLAQGHPTFLVREIT-PSNPEKAQLLESFTSKGA	56
pine-PBER	1: M-G-SRSRILLIGATGYIGRHVAKASLDLGHPTFLVREASTASSNEKAQLLESFKASGA	58
Bet v 5	1: MA--HKSILLIGGTGYIGKFIVEASAKSCHPTFALVRE-STVSDPVKGLVEKFKGLGV	57
Pyr c 5	1: MA--SKSILLIGGTGYIGKFIVEASAKAGYPTVVLVRE-ASLSDPAKSKVIENFKALGV	57
soybean-IFR	1: MAGDSKSKILLFIGGTGYIGKFIVEASAKAGHPTFLVRE-STLSNPAKSPLIDNFKGLGV	59
potato-IFR	1: MA--GKSKILLFIGGTGYIGKFIVEASAKAGHPTFLVRE-STLSNPTKTKLIDTFKSGV	57
CJP-6	57: TLVQGSIDDHASLVAAIKKVDVVISTLIGAPQIADQFNLIKAIKEVGTIKRFFPSEFGNDV	116
pine-PBER	59: NIVHGSIDDHASLVAAIKKVDVVISTVGSLOIESQVNTIKAIKEVGTIKRFFPSEFGNDV	118
Bet v 5	58: TLLHGDLYDHESLVKATKQVDVVISTVGHQLADQVKIITAIKEAGNIKRFFPSEFGNDV	117
Pyr c 5	58: NFVLGDLYDHESLVKATKQVDVVISTVGHQLADQVKIITAIKEAGNIKRFFPSEFGNDV	117
soybean-IFR	60: NLVLGDLYDHOSLSVATKQVDVVISTVGHQLADQVKIITAIKEAGNIKRFFPSEFGNDV	119
potato-IFR	58: TFVHGDLYDHESLVKATKQVDVVISTVGHALLADQVKIITAIKEAGNIKRFFPSEFGNDV	117
CJP-6	117: DKHHAVERPMKSMFLIKIKLRRTTEAEGIPHTYVPHCFAGYFLTNLAQLGLAAPPRDKIV	176
pine-PBER	119: DNVHAVERPAKSVFEVKAKVRRATEAEGIPHTYVSSNCFAGYFLRSLAQAGLTAPPRDKVV	178
Bet v 5	118: DRVHAVERPAKTAFAKAEIRRKTEAEGIPHTYVSSNFFAGYFLPTLAQGLTSPPREKVV	177
Pyr c 5	118: DRSHAVERPAKSAFETKAKIRRAVEAEGIPHTYVSSNFFAGYFLPTLNQPGASSAPPRDKVV	177
soybean-IFR	120: DRTHAVERPAKSAFATKAKVRRATEAEGIPHTYVSSNFFAGYFLPNLSQPGATAAPPRDVI	179
potato-IFR	118: DRVHAVERPAKAAENTKAIIRRVTEAEGIPHTYVATFFAGYSLPNLAQPGAAGPPNDKVV	177
CJP-6	177: IYGDGTTKAVYMKEDDIGTFTIKAVDDPRTLNTILYLPKPPANTISTINDLVALWEKKIKT	236
pine-PBER	179: ILGDGNARVVFVEEDIGTFTIKAVDDPRTLNTILYLRPANTLSINELVALWEKKIDKT	238
Bet v 5	178: IFDGNARAVFNKEDDIGTFTITRAVDDPRTLNTILYVIKPAKNIYSFNEIVALWEKKIKT	237
Pyr c 5	178: ILGDGNPKAIFNKEDDIGTFTITRAVDDPRTLNTILYVIRPPANTISNELVSLWEKKIKT	237
soybean-IFR	180: ILGDGNPKAVFNKEDDIGTFTINSVDDPRTLNTILYIRPPANTLSFNEIVTLWEKKIKT	239
potato-IFR	178: ILGDGNKAVFNKEDDIGTFTINAVDDPRTLNTILYIKPPHNTITINELVSLWEKKITGN	237
CJP-6	237: LEKVYLSEEOVLKLLQDTPFGTFMVSLHTTYVKGDQTNFQI-GPDGVEASALYPDVKY	295
pine-PBER	239: LEKAVVPEEEVLKLIADTPFPANISATSHSIFVKGDQTNFET-GPAGVEASQLYPDVKY	297
Bet v 5	238: LEKIVVPEEKLLKDIQESPIPINVLATINHSVFVKGDHTNFETEASFGVEASELYPDVKY	297
Pyr c 5	238: LERIVVPEBOLLKNIQEAAPVLNVILSTSHAVFVKGDHTNFETEPSFGVEATALYPDVKY	297
soybean-IFR	240: LERIVVPEBOLLKQIEESAPFVNVLSTINHSYVKGDHTNFETEISSFGVEASALYPDVKY	299
potato-IFR	238: LERLIVPEBOLVKNIQEASVPMNVGLSTVHTAFVKGDHTNFETEPSFGVEASEVYPDVKY	297
CJP-6	296: TTVEEYISAFV	306
pine-PBER	298: TTVDEYLSNFV	308
Bet v 5	298: TTV-----	300
Pyr c 5	298: TTVDEYLNQFV	308
soybean-IFR	300: ITVDEYLNQFV	310
potato-IFR	298: TPIDEILLNQYV	308

Fig. 3. Multiple sequence alignment of CJP-6 polypeptide with plant IFR family members including pine phenylcoumaran benzylic reductase (PBER), birch pollen allergen Bet v 5, pear allergen Pyr c 5 and IFR from soybean or potato. Identical amino acids are grey-boxed. The IFR family characteristic NA (P)DH binding motif [GX(X)GXXG [18]] is indicated by asterisks.

AF202183) or potato (60%, X92075 [20]). Intriguingly, we found that the IFR family includes plant allergens possessing IgE-binding activity; significant sequence homology was observed with recently identified birch pollen allergen Bet v 5 (61% identity, AF135127 [21]), and pear allergen Pyr c 5 (60%, AF071477).

Expression and purification of recombinant CJP-6 in *E. coli*

To examine allergenicity and immunochemical properties of CJP-6 polypeptide, we next prepared recombinant protein using GST-fusion protein expression system in *E. coli*. The 921 bp CJP-6 ORF was rescued by PCR amplification, and then inserted into pGEX-3X expression vector. Figure 4(a) presents SDS-PAGE analysis of recombinant CJP-6 expression and confirmation of its purification step. Upon IPTG induction, a 61-kDa protein was newly expressed in the BL-21 transformant carrying the expression plasmid (Fig. 4a, lane 3). Western blot analysis with anti-GST serum demonstrated that this 61 kDa molecule was recombinant GST-fusion protein (data not shown). Because the recombinant protein was observed in post-sonicated soluble cell lysate (Fig. 4a, lane 4), we next proceeded to its affinity purification. After adsorption of the 61 kDa GST-fusion protein on glutathione Sepharose 4B column, recombinant CJP-6 (r-CJP-6) was obtained by Factor Xa cleavage (Fig. 4a, lane 5). Amino acid sequencing analysis demonstrated that N-terminal 15 amino acids from the purified r-CJP-6 coincided with those predicted from the cDNA sequence (data not shown). Furthermore, we also confirmed by

western analysis that r-CJP-6 was recognized by rabbit anti 30 kDa antigen serum IgG, which is used as a probe for cDNA library screening (Fig. 4b).

Recombinant CJP-6 is recognized by IgE from Japanese cedar pollinosis patients with high frequency

To test whether r-CJP-6 can bind IgE from pollinosis patients, we first performed western blot analysis using sera from 15 patients (RAST score > 3, all of them are Cry j 1-positive as judged by ELISA; data not shown) and three non-allergic healthy individuals. We found that r-CJP-6 was recognized by patients' IgE on the blot (10/15, 67% frequency), whereas no positive signal was obtained when probed with control non-allergic sera (Fig. 5). To confirm IgE reactivity is due to IgE epitopes that occur in the natural protein and not due to an artifact, we performed competitive ELISA. We found that crude *C. japonica* pollen extract significantly inhibited IgE binding to immobilized r-CJP-6 in a dose-dependent manner, whereas irrelevant ovalbumin did not (data not shown). This demonstrates that anti-r-CJP-6 IgE determinants actually exist on their natural counterpart in the cedar pollen extract. Taken together, these data indicate that the IFR homologue in Japanese cedar pollen is a new IgE-binding protein.

To further analyse IgE binding frequency, we also applied r-CJP-6 to ELISA with sera from 25 pollinosis patients (RAST score > 3, Cry j 1-positive). We found that 76% of the patients (19/25, patient nos 5–9 and 11–24) exhibited IgE binding ability

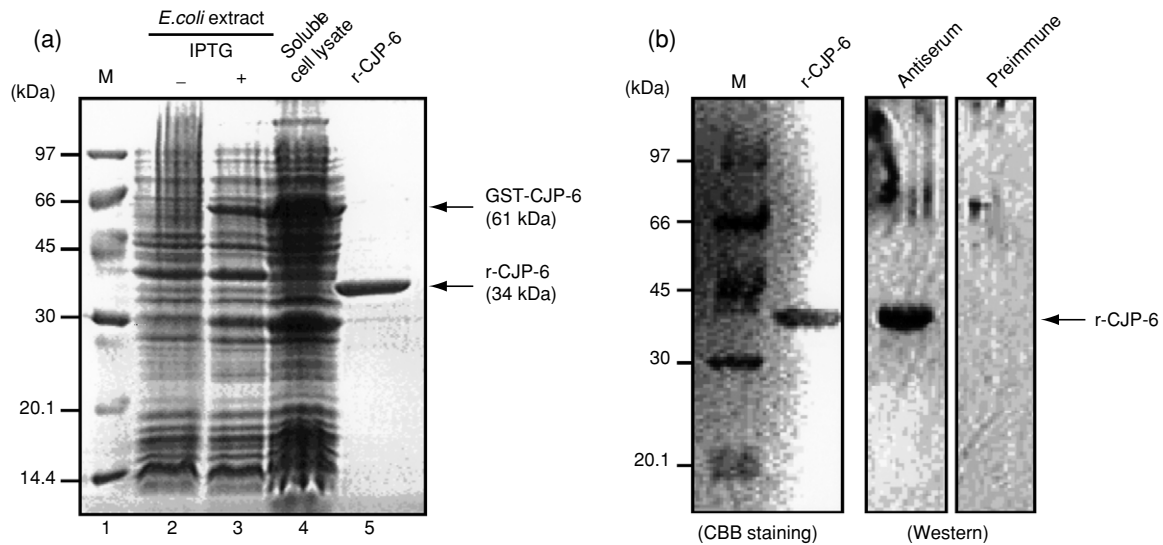


Fig. 4. (a) Expression and purification of recombinant CJP-6. *E. coli* BL-21 was transformed with pGEX-3X vector carrying the CJP-6 ORF, and recombinant GST-fusion protein was expressed upon induction with IPTG supplementation. Expression and purification steps of the r-CJP-6 were then monitored by SDS-PAGE analysis. Lane 1; molecular weight marker, lane 2; total cell extract from BL-21 transformant without IPTG addition, lane 3; cell extract from IPTG-supplemented transformant, lane 4; soluble cell lysate after sonication of IPTG-treated transformant, lane 5; purified r-CJP-6 by glutathione Sepharose 4B column chromatography. (b) Western blot analysis of r-CJP-6 with rabbit antiserum raised against 30 kDa Japanese cedar pollen antigen. Purified r-CJP-6 showed positive binding with antiserum, which was used in the immunoscreening of *C. japonica* cDNA library.

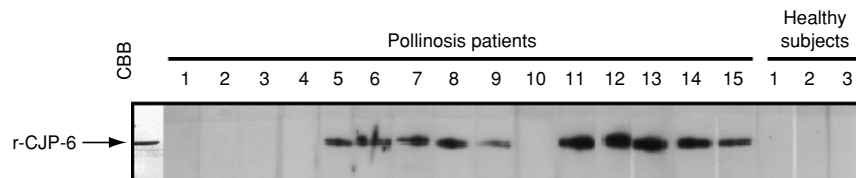


Fig. 5. Western blot analysis of r-CJP-6 with cedar pollinosis patient IgE. Purified r-CJP-6 was subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. After cutting the membrane into strips carrying equal amount of r-CJP-6, resulting blots were stained with Japanese cedar pollinosis patient sera ($n = 15$), or with those from non-allergic subjects ($n = 3$) as a control. IgE-binding to r-CJP-6 was visualized by secondary anti-human IgE antibody staining followed by ECL detection as described in Materials and methods. Protein loading was confirmed by CBB staining of the membrane strip (see the lane 'CBB').

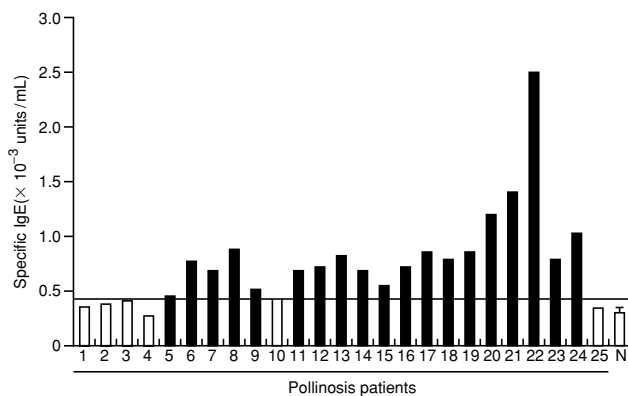


Fig. 6. High IgE-binding frequency of r-CJP-6. ELISA was performed using sera from 25 cedar pollinosis patients (RAST score > 3 , Cry j 1-positive as judged by ELISA) and three normal individuals (indicated 'N') as a negative control. A dashed line indicates a cut-off value (mean ELISA value + $3 \times$ SD from control normal sera) for judging positive IgE binding to r-CJP-6. Patients indicated by black bars (Nos 5-9 and 11-24) are r-CJP-6-positive donors.

against r-CJP-6, suggesting its high IgE binding frequency (Fig. 6). It is noteworthy that the birch pollen IFR homologue Bet v 5 has been reported as a minor allergen with moderate (9/28, 32%) IgE binding frequency [21]. Thus, in contrast to the case of birch pollen allergy, IFR homologue in Japanese cedar pollen might be an additional major allergen. Evaluation of *in vivo* allergenicity of natural CJP-6 would clarify this possibility.

Our results, together with a recent finding that the IFR-like molecule in birch pollen is a new allergen [21], further raise the possibility that the IFR-family member is a cross-reactive plant allergen. In fact, Karamloo et al. have reported that anti-Bet v 5 monoclonal antibodies recognize cross-reacting proteins in plant foods [21]. They also have identified IFR-homologous allergen from pear (Pyr c 5, GenBank Accession No. AF071477). To test whether the CJP-6 molecule shows IgE cross-reactivity with IFR counterparts from other atopy-inducing pollens and/or plant foods would be an additional important issue.

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