A New Lipid Transfer Protein Homolog Identified as an IgE-Binding Antigen from Japanese Cedar Pollen

Ahmed Ragaa Nour Ibrahim, Seiji Kawamoto, Minori Nishimura, Syunka Pak, Tsunehiro Akı, Araceli Díaz-Perales, Gabriel Salcedo, Juan A. Asturias, Takaharu Hayashi, and Kazuhisa Ono

1Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8530, Japan
2Unidad de Bioquímica, Departamento de Biotecnología, E.T.S Ingenieros Agronomos, Universidad Politecnica, Madrid, Spain
3Research and Development Department, Bial-Aristegui, Bilbao, Spain
4Takanobashi Central Hospital, Hiroshima 730-0042, Japan

Received September 18, 2009; Accepted December 25, 2009; Online Publication, March 7, 2010
[doi:10.1271/bbb.90685]

Japanese cedar (Cryptomeria japonica) pollen is a major cause of seasonal rhinitis and conjunctivitis in Japan, and an understanding of its full allergen repertoire is prerequisite for the development of future molecular diagnostics and immunotherapeutic strategies. Here we report the identification of a new C. japonica pollen IgE-binding antigen (CJP-8) homologous to lipid transfer proteins (LTPs), a class of plant cross-reactive allergens found in foods, latex, and pollen grains. The cjp-8 cDNA encodes a 165-amino acid polypeptide possessing the conserved eight cysteines characteristic of plant LTP family members. Escherichia coli-expressed recombinant CJP-8 (r-CJP-8) reacted with IgE antibody from Japanese cedar pollinosis patients at a 37.5% frequency (6/16).

Key words: allergen; lipid transfer protein; Japanese cedar pollen; oral allergy syndrome

Japanese cedar (C. japonica) pollen represents a major trigger factor in seasonal pollinosis during the winter-spring season. The prevalence of pollinosis in Japan caused by Japanese cedar pollen increased by 10% in the last decade to 26.5%, making Japanese cedar pollen the most serious allergic disorder in Japan. To develop effective diagnostic and therapeutic strategies, the identification and immunochemical characterization of responsible allergen molecules is an essential step. To date, two C. japonica pollen antigens, Cry j 1 (pectate lyase) and Cry j 2 (polymethylgalacturonase), have been identified and extensively studied as major allergens,5-13 while the identities of other allergen molecules are poorly understood.

We have reported the existence of many uncharacterized IgE-reactive antigens in C. japonica pollen extract, and have identified the third major allergen, CJP-6, belonging to the isoflavone reductase family of allergens.6 We also have identified a total of 131 distinguishable IgE reactive spots on a two-dimensional (2-D) IgE immunoblotting map of C. japonica pollen extract,7 on which 40 spots are major allergens with higher IgE-binding frequency than that of Cry j 2. Among those antigen spots, we have cloned cDNA encoding another major allergen, CJP-4, a class-IV chitinase homolog showing a very potent (100%) IgE-binding frequency as well as IgE cross-reactivity with latex C-serum.8

Plant non-specific LTPs are a family of basic polypeptides (pI around 9) with molecular sizes of 9–15 kDa.9-10 This family exhibits various degrees of sequence identity (from 30% to 95%), but all members possess eight conserved cysteines forming four disulfide bonds, which are responsible for LTP compact folding.10 Plant non-specific LTPs (nsLTPs) also constitute a major class of plant allergens, found in food, latex, and pollen.9-10 This allergen family might be pathophysiologically important, because its interspecies cross-reactivity can evoke the food allergy called oral allergy syndrome (OAS), in which patients suffer from allergic symptoms such as oral swelling, itching, urticaria, and systemic anaphylaxis upon intake of the foods (and/or exposure to pollen) containing cross-reactive allergens.11-13 Indeed, OAS cases potentially associated with Japanese cedar pollinosis have been reported,14-16 but the responsible allergens are currently unknown. Molecular characterization of those cross-reactive allergens is critical in the prevention and treatment of pollinosis-related OAS.

Here we report the cDNA cloning and characterization of a novel class of Japanese cedar pollen IgE-binding antigen CJP-8 structurally belonging to plant LTP.

Materials and Methods

Japanese cedar pollen, reagents, and pollinosis patient sera. C. japonica pollen was collected at Mikado island in Hiroshima Prefecture, Japan, and stored at −80°C until used in antigen preparation or cDNA library construction. General-use chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

1 To whom correspondence should be addressed. Tel: +81-82-424-7753; Fax: +81-82-424-7755; E-mail: skawa@hiroshima-u.ac.jp
A. R. N. I. and S. K. contributed equally to this work.
Abbreviations: LTP, lipid transfer protein; OAS, oral allergy syndrome; PBS, phosphate-buffered saline
Katayama Chemical (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), and Wako Pure Chemical Industries (Osaka, Japan) unless specifically indicated otherwise. Serum samples were collected from Japanese cedar pollinosis patients whose CAP-RAST scores were over 4 and whose IgE-binding abilities to major allergen Cry j 1 were positive. Control non-allergic sera were collected from healthy volunteers at Hiroshima University. These sera were stored at −30°C until use. All clinical experiments were approved by the Institutional Review Board of Takanobashi Central Hospital, and were described in detail to all participants, who provided informed consent.

Preparation of Japanese cedar pollen extract. Pollen (80g) was stirred in 3.2 liters of phosphate-buffered saline (PBS) at 4°C for 4h, and the homogenate was centrifuged at 6,000 rpm for 30min. The supernatant was applied to ammonium sulfate precipitation (80% saturation), and the resulting precipitate was dissolved in distilled water followed by overnight dialysis at 4°C. After centrifugation (18,800 g for 30 min), the supernatant was filtered with a 0.22-μm filter unit (Millipore, Bedford, MA) to obtain crude pollen extract. The extract was freeze-dried and stored at −80°C until use.

Construction of a C. japonica pollen cDNA library. Extraction of total RNA, selection of poly(A+) RNA, and synthesis of double-stranded cDNA were carried out as described previously.61 cDNA library was constructed on phage vector λZIPOLEX (Invitrogen, Carlsbad, CA) by inserting the double-stranded cDNA into the Not I/Sal I site of the vector arm.

Cloning of cjp-8 cDNA. The pollen cDNA library (5 × 10^8 pfu) was screened with PCR-amplified cDNA from Japanese cedar pollen patients, and isolated positive cDNA clones were recovered as phagemids (pZL1) by Cre/loxP system-mediated in vivo excision in the Escherichia coli (E. coli) DH12S strain. Both strands of the cDNA were then sequenced using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). We chose cDNA clone cjp-8 for further analysis in view of its significant sequence identity to that of pollen LTPs. cDNA templates for Rapid Amplification of cDNA Ends (RACE)-polymerase chain reaction (PCR) were synthesized using the BD SMART RACE cDNA Amplification Kit (BD Biosciences, San Jose, CA). 5′- and 3′-RACE PCR was then carried out using the same kit with cjp-8-specific primers for 5′-RACE (5′-CACTAGGGCTTTGTCGAAACAAGC-3′) and 3′-RACE (5′-CGAAACCCCCGGATTCGCCGTTGG-3′). The resulting PCR fragments were subcloned into pGEM-T Easy Vector (Promega, Madison, WI) and their nucleotide sequences were determined with an ABI PRISM 310 Genetic Analyzer.

Expression and purification of recombinant allergens. A cDNA fragment encoding full-length CJP-8 polypeptide (nucleotide no. 255255) was prepared by PCR using specific primers (forward 5′-GAAACATATGCGGAGATGAGAATGAAACG3′; reverse 5′-GGTAGCCATTGAGGGATGTTTGAACACG-3′; Nde I and Xho I restriction sites were underlined). The resulting PCR fragment was digested with Nde I and Xho I and then subcloned into pCold TF cold shock expression vector (TAKARA Bio, Otsu, Japan). The E. coli Rosetta-gami (DE3) pl4 strain (Novagen, Madison, WI) was transformed with the expression plasmid, and the transformant was cultured at 37°C in 2 L of YD medium supplemented with 15 μg/ml of kanamycin, 34 μg/ml of chloramphenicol, 15.5 μg/ml of tetracycline, and 50 μg/ml of streptomycin until it reached a cell density of OD 600 = 0.5. Then the culture was cooled at 15°C for 30 min, and further cultured at 15°C for 24 h in the presence of 1.0 mM isopropyl-1-thio-β-D-galactoside (IPTG) to induce the expression of recombinant CJP-8 (r-CJP-8) as a trigger factor (TF)-fusion protein. r-TF-CJP-8 fusion protein was purified by metal chelate affinity chromatography using a HisTrap HP column equipped with AKTA Explorer 10S (GE Healthcare Bio-Sciences, Uppsala, Sweden). The TF-CJP-8 was eluted with a linear gradient of 40–500 mM imidazole and desalted using a PD-10 column (GE Healthcare Bio-Sciences). After it was freeze dried, the lyophilized fusion protein was dissolved in 150 mM NaCl/2.5 mM CaCl2/20 mM Tris-HCl (pH 8.37) and digested with thrombin (Novagen) to remove the TF-tag. The non-fusion r-CJP-8 band was finally excised from SDS-PAGE gels (see below for details of protein electrophoresis procedures), and stained with a Negative Gel Staining MS Kit (Wako Pure Chemical Industries, and the recombinant protein was extracted from the gel upon overnight incubation in 1% SDS/Tris-HCl (pH 8.0) at 4°C. For N-terminal amino acid sequencing of the recombinant proteins, the proteins, separated in SDS–PAGE gels, were electrophoblated onto a polyvinylidene difluoride filter (Immobilon P, Millipore). After staining of the membrane with 0.25% Coomassie Brilliant Blue (CBB) R250, the corresponding protein bands were excised, and the N-terminal amino acid sequence was determined using a 492 Protein Sequence (Applied Biosystems). Pru p 3 (peach LTP allergen)17,18 and r-Par j 1 (Parietaria judaica pollen LTP allergen)19,20 were expressed as recombinant proteins in the methyl-trophic yeast Pichia pastoris and prepared as described previously.21,22

SDS–PAGE and Western blot analysis. Protein samples were resolved by SDS–PAGE by the discontinuous buffer system of Laemmli23 on a 12.5% acrylamide slab gel. Fractions of proteins were then visualized by 0.25% CBB R250 staining or silver staining. For immunoblotting, the fractionated protein was transferred electronically onto an Immobilon P membrane (Millipore). The (His)6-tagged recombinant TF fusion protein was detected by staining with murine anti-His monoclonal antibody (1:20,000, GE Healthcare Bio-Sciences) followed by reaction with secondary horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG polyclonal antibody (1:20,000, Sigma-Aldrich). To test IgE-binding capacity, the blots were incubated with pooled sera from Japanese cedar polinosis patients (1:10 dilution) and then stained with secondary biotinylated goat anti-human IgE antibody (Vector Laboratories, Burlingame, CA) followed by reaction with HRP-conjugated streptavidin (Zymed, San Francisco, CA). Protein bands were visualized on X-ray film (Konica, Tokyo) using ECL Plus Western Blotting Detection Reagent (GE Healthcare Bio-Sciences).

Enzyme-linked immunosorbent assay (ELISA). The IgE-binding frequency of r-CJP-8 was evaluated by ELISA, as previously described,61 with minor modifications. In brief, 50 μl of purified r-CJP-8 (2 μg/ml in 100 mM bicarbonate buffer, pH 9.4) was coated onto a 96-well microtiter plate (NUNC-ImmuNo Maxisorp P96, Nalge Nunc International, Roskilde, Denmark), and incubated at room temperature for 2hr. After incubation of the plate with a blocking buffer (3% skim milk/1% bovine serum albumin/PBS) overnight at 4°C, 50 μl of diluted Japanese cedar pollenosis-patient sera (diluted 1:10 with blocking buffer) or those from control healthy donors were added and incubated for 2hr at room temperature. Then 50 μl of biotinylated goat anti-human IgE (Vector Laboratories, 1:10,000) was added, and the plate was incubated for 2hr at room temperature, followed by incubation with 50 μl of alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA, 1:1,000) for 1hr. Finally, 100 μl of p-nitrophenyl phosphate substrate solution (Promega) was added, and fluorescence intensity of each well was measured using a Wallac 1420 ARVOx Multilabel Counter (Perkin Elmer Life Sciences, Boston, MA). The cutoff ELISA value of positive IgE-binding was calculated as the mean ELISA value + 3 x standard deviation (SD) obtained from healthy donors (n = 8).

Competitive ELISA. A 96-well microtiter plate was coated with r-CJP-8 (5 μg/ml in 100 mM carbonate buffer) and blocked by the above-described procedures. Prior to reaction with coated r-CJP-8, individual cedar polinosis patient sera (×10 dilution) were pre-incubated at room temperature for 2hr with serial dilutions of competitors (r-CJP-8, peach allergen r-Pru p 3, or P. judaica allergen r-Par j 1, with C. japonica major allergen Cry j 2 as a negative control). The pre-adsorbed sera were then applied to the r-CJP-8-coated microtiter plate to test IgE cross-reactivity. Subsequent secondary antibody staining and signal detection were carried out as described above.

Results and Discussion

cDNA cloning and structural analysis of a new Japanese cedar pollen IgE-binding antigen homologous to plant LTP

Immunoscreening of a C. japonica pollen cDNA library with Japanese cedar pollinosis-patient IgE antibodies yielded several positive clones. Among these, we focused on a cDNA clone, cjp-8, since its nucleotide
sequence showed significant homology to those of plant LTPs. We then obtained cjp-8 cDNA containing an open reading frame using 5′- and 3′-RACE PCR. Sequence analysis revealed that the cjp-8 cDNA comprised 927 bp nucleotides encoding a 165-amino acid polypeptide (Fig. 1) with a calculated molecular weight and isoelectric point of 17,409 Da and 7.39. Its N-terminal 35 amino acids were hydrophobic and were predicted to be a signal peptide (as analyzed by the SignalP 3.0 software, shown underlined in Fig. 1). The putative mature polypeptide had eight cysteines (shown gray boxed in Fig. 1), and its number was identical to those seen in plant LTP family members. BLAST amino acid similarity search indicated that CJP-8 polypeptide exhibited significant sequence identity to the plant protease inhibitor/seed storage/LTP family of proteins. The highest homology score was noted as compared with LTP (PA18) from the Norway Spruce tree Picea abies (44.7% identity/123 amino acid overlap, Genbank/EMBL/DDBJ accession no. BAA23548.1) and Medicago truncatula LTP/Par allergen (44.1%/111 amino acids, Q1SBU5) (Fig. 2A). Another set of multiple alignments with known plant LTP allergens indicated that the CJP-8 polypeptide was less homologous to this family of allergens (19.8–28.8%/66–98 amino acid overlap), although partial sequence identity was observed, particularly in the N-terminal region (residues 43–53, see Fig. 2B). However, the alignments clearly showed the eight cysteines completely conserved, potentially forming four disulfide bridges, which were responsible for the LTP compact folding (shown by black boxes and asterisks in Fig. 2B). Taken together, these results suggest that CJP-8 polypeptide belongs structurally to the plant LTPs.

Expression and purification of recombinant CJP-8 (r-CJP-8)

To analyze immunochemoical properties, we next sought to express r-CJP-8 using E. coli cold-shock expression vector pCold TF, which enabled soluble protein expression via fusion with trigger factor (TF) as a chaperone molecule. SDS–PAGE and western blotting with anti-His mAb indicated successful expression of TF-CJP-8 fusion protein (77.5 kDa, shown in Fig. 3A and B, lane 2). Furthermore, western blot analysis showed that r-TF-CJP-8, but not the control r-TF-tag, was recognized by IgE antibodies from Japanese cedar pollinosis patients (data not shown), indicating that CJP-8 is a novel Japanese cedar pollen allergen. Next we tried to purify non-fusion r-CJP-8. First we first purified r-TF-CJP-8 fusion protein by HisTrap metal chelate affinity chromatography (Fig. 3, lane 3). Subsequent thrombin digestion of TF-CJP-8 yielded a 57 kDa TF-tag and two protein bands around 20 kDa (indicated by small arrowheads in Fig. 3A, lane 4). Protein sequencing revealed that the N-terminal amino acid sequences of both 20 kDa bands completely matched those deduced from the cjp-8 cDNA sequence (data not shown). Thus we finally purified the upper major r-CJP-8 band (20.5 kDa) by non-reducing SDS–PAGE and gel extraction procedures (Fig. 3A, lane 5).

IgE-Binding capacity of r-CJP-8

To determine the IgE-binding capacity of CJP-8, we applied the purified r-CJP-8 to ELISA with 16 Japanese cedar pollinosis patient sera (CAP-RAST score ≥ 4 and Cry j 1-positive). Sera from eight healthy donors were also used as a negative control. We found that six to 16 pollinosis donors (nos. 1, 6, 7, 10, 12, and 15) exhibited positive IgE-binding to r-CJP-8 (Fig. 4), as judged by the observation that their CJP-8-specific IgE titers were higher than a cutoff value (mean IgE titer + 3SD calculated from the healthy donors, indicated by a dotted line). These results clearly indicate that this LTP homolog represents a new class of C. japonica IgE-binding antigen. The low IgE-binding frequency (37.5%) against the biased pollinosis donors (all of the selected patients showed positive IgE-binding to Cry j 1) suggests that CJP-8 is a minor allergen in Japanese cedar pollinosis. Further analysis is needed to determine in more detail the IgE-binding potency and allergenicity of CJP-8 using natural allergen.
Fig. 2. Multiple Sequence Alignment of CJP-8 Polypeptide with Plant LTPs and LTP Allergens.

A. Alignment of CJP-8 with highly homologous LTPs from Norway Spruce *P. abies* (Genbank/EMBL/DDBJ accession no. BAA23548.1) and *M. truncatula* LTP/Par allergen from *M. truncatula* (Q1SBU5). Identical amino acids are shown black boxed (within all three members) or gray boxed (between two polypeptides). Asterisks indicate the conserved cysteines. B. Multiple alignment with plant LTP family members, which include peach Pru p 3 (P81402), apple Mal d 3 (CA9B6874), strawberry Fra a 3 (AAY83346), orange Cit s 3 (Q6EV47), tomato Lyc e 3 (CAJ19705), hazelnut Cor a 8 (AAK28533), maize Zea m 14 (P19656), latex Hev b 12 (AAL25839), and *P. judaica* pollen Par j 1 (O04404). The conserved cysteine residues are shown black boxed/asterisked, and the other identical amino acids are gray boxed.

Fig. 3. Expression and Purification of r-CJP-8 in *E. coli*.

*E. coli* Rosetta-gami cells were transformed with cold shock expression vector pCold TF carrying the CJP-8 open reading frame, and r-CJP-8 expressed as a TF fusion protein was purified by HisTrap metal chelate affinity chromatography. After digestion with thrombin, cleaved r-CJP-8 was obtained from the SDS–PAGE gel by the gel extraction procedure described in “Materials and Methods.” A, SDS–PAGE inspection of expression and purification steps. Lane 1, cell lysate from the control pCold TF transformant; lane 2, lysate from the CJP-8 transformant; lane 3, semi-purified r-TF-CJP-8 by the HisTrap metal chelate affinity column; lane 4, thrombin digest of r-TF-CJP-8; lane 5, purified r-CJP-8. B, Recombinant TF fusion proteins (r-TF-CJP-8 or free TF) in each sample were visualized by western blotting using anti-His mAb.
IgE cross-reactivity between r-CJP-8 and other plant LTP allergens

The plant LTP allergens from fruits and pollens show interspecies IgE cross-reactivity, and are thought to be involved in the pathogenesis of pollinosis-related OAS.9,10 Although the CJP-8 polypeptide is less homologous to plant LTP allergens, partial sequence identity is seen, particularly in the N-terminal portion around the second conserved cysteine (residues 43–53, Fig. 2B). In addition, the complete conservation of cysteine residues implies a possible conformational similarity among these molecules. These facts prompted us to test whether CJP-8 would show IgE cross-reactivity with other plant LTP allergens. To address this question, we performed competitive ELISA using peach major allergen Pru p 318 and Parietaria judaica pollen allergen Par j 119,20 as model LTP competitors.

Among the four CJP-8-positive cedar pollinosis donors tested, we found one patient whose CJP-8-specific IgE antibody cross-reacted to r-Pru p 3 as well as to r-Par j 1 to a lesser extent (Fig. 5), while the remaining pollinosis donors did not (data not shown). In this positive donor, r-Pru p 3 dose-dependently suppressed IgE-binding to r-CJP-8 (80% inhibition), whereas the addition of r-Par j 1 resulted in a moderate (50–60%) inhibition of anti-CJP-8 IgE immunoreactivity (Fig. 5). The negative control cedar pollen allergen, Cry j 2, showed no sign of cross-reactivity, and a positive control inhibition curve using r-CJP-8 showed full competition over an antigen dose of 2,000 pmol, indicating that the overall competition assay was well controlled. Taken together, these data raise the possibility that CJP-8 works as a cross-reactive LTP antigen, but the prevalence of the IgE cross-reactivity was low (1 to 4 CJP-8-positive donors, 25%) in this experiment. Thus further clinical studies with increased patient cases are essential to confirm whether CJP-8 shows IgE cross-reactivity against some Japanese cedar pollinosis patients.
The structural basis of the observed IgE cross-reactivity is unknown. In the case of Pru p 3, one plausible assumption is that the anti-CJP-8 IgE linear epitope overlapped that residing in Pru p 3 in this patient. One candidate for such an epitope region is the N-terminal portion of CJP-8 (residues 43–53), which shows partial amino acid similarity to Pru p 3 (Fig. 2B). Another possibility is the existence of common conformational IgE epitopes among these LTP family members in spite of their low sequence similarities. Further analysis of their structure-activity relationship is needed to address these questions.

Conclusions
In summary, we identified and characterized a novel Japanese cedar pollen IgE-binding antigen (CJP-8) structurally belonging to the plant LTP family, a class of plant cross-reactive allergens. The results presented here perhaps throw light on the development of future component-resolved molecular diagnosis of Japanese cedar pollinosis as well as its immunotherapeutic intervention. In addition, r-CJP-8 should be an useful tool to test whether plant LTP family members play pathogenic roles in pollinosis-related oral allergy.

References