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Purification, Identification, and cDNA Cloning of Cha o 2, the Second Major Allergen of Japanese Cypress Pollen

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The second major allergen of *Chamaecyparis obtusa* (Japanese cypress) pollen, Cha o 2, has been purified and its cDNA cloned. Of patients with pollinosis caused by *C. obtusa*, 82.5% produce IgE antibodies which react with purified Cha o 2. The purified protein has a molecular mass of 46 kDa and its 12 N-terminal amino acid sequence displays a high homology with that of Cry j 2, the second major allergen of *Cryptomeria japonica* pollen. cDNA clones coding for Cha o 2 have been isolated using Cry j 2 cDNA as a probe. Cha o 2 cDNA clones were sequenced and found to code a putative 50-residue signal sequence and a 464-residue mature protein with a molecular weight of 50 kDa. Two possible N-linked glycosylation sites were found in the sequence. The deduced amino acid sequence of Cha o 2 shows 74.3% identity with that of Cry j 2. In its primary structure, Cha o 2 shows significant identity with those of the polygalacturonases of avocado, tomato, and maize as well as Cry j 2.

Allergic disease is an important public health problem, especially in developed countries. Pollen of *Chamaecypris obtusa* (Japanese cypress, Hinoki) of the Cupressaceae family is one of the most important factors related to respiratory allergies in the world, and is also one of the most serious aeroallergens in Japan.

Similarly, the pollen of *Cryptomeria japonica* (Japanese cedar, Sugi) is a well known spring aeroallergen in Japan. The geographical range on area which *C. obtusa* is found or has been planted, however, has recently been expanding in Japan. Therefore, pollinosis caused by *C. obtusa* is likely to increase.

Many patients with pollinosis in Japan have specific Ig E antibodies not only to *C. japonica* but also to *C. obsuta* (1, 2). We speculated that there might be close cross-reactivity between these species.

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Cry j 1 and Cry j 2 are two allergen molecules of *C. japonica* with molecular masses of 41–45 kDa and 37 kDa, respectively (3). cDNA cloning has recently revealed the primary structure of these molecules (4, 5).

In the molecular analysis of *C. obtusa*, Cha o 1, one of the major allergens, was purified and its cDNA cloned (6). Using the results of a previous study, amino acid sequence of Cha o 1 showed about 80% homology with that of Cry j 1, nevertheless the biological classification was different. Here we report the purification of the second major allergen, designated Cha o 2, using an anti-Cry j 2 rabbit serum and the cDNA cloning by cross-hybridization with a Cry j 2 cDNA fragment. The information on the primary structure of Cha o 2 should be useful in the determination of T-cell epitopes and for the development of a cure for pollinosis caused by *C. obtusa*.

MATERIALS AND METHODS

Purification and sequence analysis of Cha o 2 protein. 30 g of pollen from *C. obtusa* was defatted five times with 60 ml of diethylether and dried overnight at room temperature. Defatted pollen was suspended in 900 ml of 125 mM $NaHCO_3$ and sonicated with SONIFIER cell disruptor for 15 minutes on ice. The homogenate was stirred overnight at 4°C. Crude extract was obtained by centrifugation at 22,000 xg for 30 minutes. 800 ml of the extract was applied to a DE52 anion-exchange cellulose column (130 ml bed; Whatman, U.K.), which had been equilibrated with 20 mM Tris-HCl (pH 7.0). The DE-52 column was further washed with 150 ml of 20 mM Tris-HCl (pH 7.0) and these pass-through were combined, and adjusted to 0.5 M NaCl by the addition of solid salt.

The fraction containing 0.5 M NaCl was applied to a Chelating Streamline column (50 ml bed; Pharmacia) at 4°C, which had been equilibrated Zn^{2+} buffer. The column was washed with 200 ml 0.5 M NaCl/20 mM Tris-HCl (pH 7.0) buffer. Elution was carried out with three 150 ml solutions of 0.5 M NaCl/20 mM sodium acetate buffer, which had been adjusted to pH 6.0, 5.0 and 4.0, respectively. By the EIA assay, the Cha o 2 protein was found to elute at pH 4.0. The pH 4.0 eluates containing Cha o 2 were pooled (36 ml) and dialyzed overnight against 2,000 ml of 10 mM NaOAc (pH 4) at 4°C. The dialysate was further purified on a Mono S HPLC column (Pharmacia). The elution was conducted using a 0–0.5 M NaCl linear gradient in 10 mM NaOAc buffer (pH 4). For further purification, hydrophobic chromatography was performed with HiTrap Phenyl Sepharose HP (Pharmacia, Sweden). Post-Mono S fraction was dia-

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lyzed overnight against 500 ml of 2 M $(NH_4)_2SO_4$/50 mM phosphate buffer (pH 7.2) at 4°C and the dialysate was applied to a HiTrap Phenyl Sepharose HP column, which was previously equilibrated with 2 M $(NH_4)_2SO_4$/20 mM phosphate buffer (pH 7.2). Cha o 2 was eluted using a linear reducing gradient from 2 M to 0 M $(NH_4)_2SO_4$/20 mM phosphate buffer (pH 7.2). Cha o 2 fractions were confirmed by EIA and the purity of Cha o 2 was determined by SDS-PAGE following silver staining.

In order to determine of the N-terminal amino acid sequences, the purified Cha o 2 protein was subjected to SDS-PAGE and transferred to a PVDF membrane (Immobilon-P_{SQ}, Millipore). The filter was stained with Coomassie Brilliant Blue R-250 and the Cha o 2 protein band was excised from the filter and used for direct protein microsequencing (HPG1005A Protein Sequencing System).

Detection of Cha o 2 Protein by Western blotting and EIA. Crude extract of cypress pollen or purified protein was subjected to SDS-PAGE (12.5%) and electro-transferred to a PVDF membrane. After blocking overnight with 3% BSA/1% NP-40/PBS, the membrane filter was incubated for 2 hours with 3,000-fold diluted anti-Cry j 2 rabbit serum. Following several washings, the filter was incubated for 1 hour with 5,000-fold diluted horse radish peroxidase conjugated anti-rabbit IgG. The enhanced chemiluminescence (ECL) detection system (Amersham) was used for detection. In some cases, 10,000-fold diluted anti-recombinant Cha o 2 rabbit serum was also used to confirm the cross-reactivity.

For EIA detection of Cha o 2 protein during the purification steps, 96-well microtitier plates (Covalink, Nunc) were coated overnight with 100 μl aliquot of diluted column fractions at 4°C. After blocking for 2 hours with the above blocking reagent at room temperature, the wells were incubated for 2 hours with anti-Cry j 2 rabbit serum and then 1 hour with HRP-conjugated anti-rabbit IgG. Cha o 2 protein was detected using TMB (3,3′,5,5′,-Tetramethylbenzidine, DAKO) as the HRP substrate.

Protein concentration determination. The protein concentration was determined using a BCA Protein Assay Reagent (Pierce, Rockford, IL) according to the manufacturer's protocol.

Isolation of RNA. Total RNA was isolated from *C. obtusa* pollen by the phenol extraction method as previously reported (5). Poly(A)+ RNA was obtained using oligo (dT)-cellulose (Pharmacia).

Northern blotting. Total RNA and mRNA were separated in 1% formaldehyde-agarose gels under denaturing conditions (7), and blotted onto a Hybond-N filter (Amersham). The filters were prehybridized at 65°C for 1 hour in a solution consisting of 10X Denhardt's solution (50X solution contains 1% Ficoll, 1% polyvinylpyrrolidone, and 1% BSA) and 4X SSC (20X solution is 3 M NaCl, 0.3 M sodium citrate, pH 7.0). Hybridization was performed at 30°C for 18 hours in a solution (4X SSC, 10X Denhardt's solution) containing a [^{32}P]-labelled Cry j 2 cDNA as a probe. The filters were washed at 30°C for 30 minutes in 2X SSC, 0.1% SDS and for 30 minutes in 1X SSC, 0.1% SDS. The filters were then autoradiographed at −80°C for 14 hours and the bands were detected on the X-Omat film (Kodak) after development.

cDNA library screening and sequencing. The lambda gt10 cDNA library, constructed with *C. obtusa* poly(A+) RNA by the same method of Sone *et al* (5), was screened using a [^{32}P]-labeled Cry j 2 cDNA fragment as a probe, which was prepared by *Eco*RI digestion of pCCII1-4 (4).

The hybridization was performed in 5X SSPE, 5X Denhardt's, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA, and 1 pmol of [^{32}P]-labeled DNA fragment at 48°C for 14 hours. Filters were washed in 2X SSPE, 0.1% SDS for 30 minutes at room temperature and the positive clones were detected by autoradiography.

The positive clones were selected and the insert DNA sequences were determined by the fluorescence-based dideoxy sequencing method using a 373A DNA sequencer (Applied Biosystems Inc., CA).

Expression of recombinant Cha o 2 protein in E. coli. To produce the recombinant His-tagged Cha o 2 protein, an expression plasmid, p-ΔNchao2 was constructed. A Cha o 2 cDNA fragment was amplified from pBSch2-5, which included full length Cha o 2 cDNA, by PCR with CH2BAM (5′-CGCGGATCCGCTGCCACCGTCTTCA-ATGTG-3′) and CHAC (5′-CGCTGCAGTTATGGATTGTAGAT-TTTGTTA-3′) primers, and *Bam*HI and *Pst*I sites were introduced on its 5′- and 3′-end, respectively. The amplified Cha o 2 fragment was subcloned into pQE9 expression vector (QIAGEN) through their *Bam*HI and *Pst*I sites. pΔNChao2 was introduced in the host *E. coli*, M15 (pREP4), for expression. Protein synthesis was induced by the addition of IPTG (final concentration 2 mM) when OD_{600} reached at 0.8. After 4 hours culture at 37°C, the cells were collected by centrifugation. The His-tagged Cha o 2 protein was purified using a Ni-NTA agarose (QIAGEN).

Fluorometric assay for IgE bound to native Cha o 2 protein. Sera from 40 allergic patients with pollinosis caused by *C. obtusa* (measured by AlaSTAT) and 16 non-allergic individuals were used and all patients and healthy individuals gave informed consent. One microgram of purified Cha o 2 protein was coated in each well on 96 well black plates (Nunc) at 4°C for 16 hours and the wells were blocked with Block Ace at 37°C for 2 hours.

Ten microliters of human serum was diluted to 100 μl with Block Ace, added to each well, and incubated at 37°C for 4 hours. The following procedure was performed according to the method of Kato *et al.* (8). The IgE bound to the solid-phase antigen was detected by β-galactosidase-conjugated anti-human IgE (Pharmacia). 4-methylumbelliferyl-β-galactopyranoside was used as an enzyme substrate and the increase of fluorescence was detected by Titertek Fluoroscan II (Flow Laboratories).

RESULTS AND DISCUSSION

Purification and characterization of Cha o 2. In order to demonstrate whether a Cry j 2 homologue protein could be detected, as Cha o 1 and Cry j 1 homologue proteins had been detected before, we carried out an immunoblot of the extract of *C. obtusa* pollen using anti-Cry j 2 rabbit serum. *C. obtusa* pollen was defatted and extracted overnight at 4°C with $NaHCO_3$ buffer. The extract was passed through a DE-cellulose column, and the eluate or crude extract was separated by SDS-PAGE and transferred to a PVDF membrane. By immunoblot with ×1,000 diluted rabbit anti-Cry j 2 serum, a 50 kDa crossreactive-band was detected and tentatively designated Cha o 2 (data not shown). To isolate the Cha o 2 protein, we adopted Zn^{2+}-chelate and hydrophobic chromatography in addition to the usual purification method of Cry j 2. For the Zn^{2+}-chelate chromatography, the Cha o 2 protein was absorbed onto the column and eluted at pH 4, while Cha o 1 protein did not bind. The result of Zn^{2+}-chelate chromatography suggested that Cha o 2 contains more histidine and cysteine residues than Cha o 1, as is the case with Cry j 1 and Cry j 2. The molecular mass of purified Cha o 2 protein on the SDS-PAGE was found to be about 50 kDa which was similar to the size of Cry j 2 (45 kDa). Starting with 30 g of *C. obtusa* pollen, we obtained 0.51 mg of Cha o 2 after purification, which is only about 1/10th the amount of Cha o 1 we obtained from an equivalent amount of pollen (6).

The purity of Cha o 2 was verified by SDS-PAGE and silver staining (Fig. 1, lanes 1 and 2). Western blotting revealed that both Cry j 2 and Cha o 2 are recognized

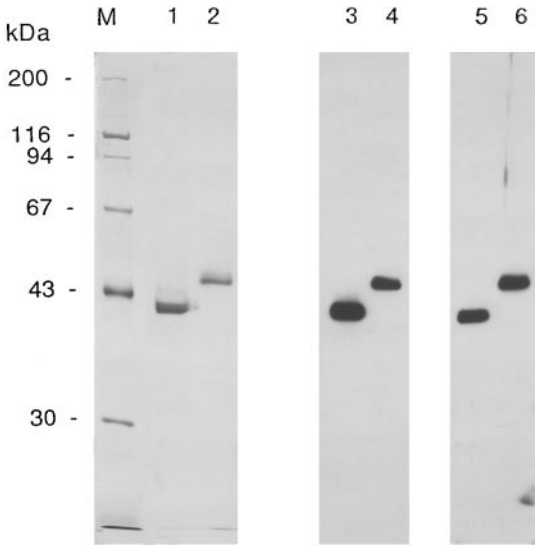

FIG. 1. Detection of Cry j 2 and Cha o 2 proteins by staining and Western blotting 250 ng of Cry j 2 (lanes 1, 3, and 5) and Cha o 2 (lanes 2, 4, and 6) were subjected to SDS-PAGE (12.5%) and were detected by silver staining method (lanes 1 and 2). The proteins in the gel were transferred to a PVDF membrane and immuno-detected with anti-Cry j 2 rabbit serum (lanes 3 and 4) or anti-Cha o 2 rabbit serum (lanes 5 and 6) using ECL system, respectively.

by anti-Cry j 2 antiserum (Fig. 1, lanes 3 and 4). Anti-recombinant Cha o 2 antiserum also recognized Cry j 2 and Cha o 2 proteins (Fig. 1, lanes 5 and 6).

The N-terminal 12 amino acid sequence of Cha o 2 was determined to be Ser-Arg-His-Asp-Ala-Ala-Thr-Val-Phe-Asn-Val-Glu. There was a correlation in the N-terminal amino acid sequences between Cha o 2 and Cry j 2 (5/8 amino acids were identical). This result showed that the N-terminal of Cha o 2 was 4 amino acids longer than that of Cry j 2. This heterogeneity between cedar and cypress may be due to the action of a different signal peptidase during secretion pathway.

Detection of specific IgE to purified Cha o 2 in human sera. The allergenicity of Cha o 2 was confirmed by testing its reactivity with human IgE. Sera of 33 (82.5%) among 40 patients reacted with Cha o 2, whereas sera of 16 healthy individuals showed no detectable reaction, from which we tentatively determined the cut off value for positive and negative IgE binding at 10 relative fluorescence. A significant difference was observed in the fluorescence values between two groups of patients and healthy individuals (308 ± 411 vs 0.250 ± 0.577, $p < 0.1\%$, Fig. 2).

In addition to Cha o 1 which we have recently reported as a major allergen in the pollen of *C. obtusa*, Cha o 2 is also one of the major allergens in the pollen of *C. obtusa* since 82.5% of the patients had specific IgE to Cha o 2 protein. However, Cha o 2 does not cause the same degree of antigenicity as Cha o 1, because 97.5% of the patients had IgE which bound to Cha o 1. Furthermore, the fluorescence value of IgE binding to Cha o 1 (data not shown) was higher than that to Cha o 2 (more than double) in the simultaneous experiment shown in Fig. 2. Therefore, Cha o 1 appears to be the primary major allergen and Cha o 2 the second major allergen in the pollen of *C. obtusa*. We have detected no correlation between the fluorescence values of anti-Cha o 1 and anti-Cha o 2 IgE antibodies in patients (data not shown) whereas some correlation between the levels of anti-Cry j 1 and anti-Cry j 2 IgE was reported (9). This difference may have been caused by a lower titer of anti-Cha o 2 IgE than that of anti-Cry j 2 IgE.

Molecular cloning of Cha o 2 cDNA and its sequence. Because of the cross-reactivity of anti-Cry j 2 serum, we thought that there might also be some homology between the Cha o 2 and Cry j 2 genes. To confirm this assumption, northern blotting was carried out using a pCCII1-4 *Eco*RI fragment as a probe, which included the part of Cry j 2 cDNA cloned by Komiyama *et al.* (4). With 3 micrograms of poly(A)+ RNA from the cypress pollen, a 1.3 kb band was detected almost the same size as that of the cedar RNA (data not shown).

For the isolation of Cha o 2 cDNA, the lambda gt10 cDNA library of cedar RNA was screened using a [^{32}P]-labelled Cry j 2 cDNA fragment as a probe. Three independent clones were isolated and sequenced. The longest clone, named CHII-10, was 1772 nucleotides.

ATG start codon was at position 32 and TAA termination codon was at 1574. The open reading frame consisted of 1542 nucleotides and coded 514 amino acids. This clone was confirmed to be Cha o 2 cDNA,

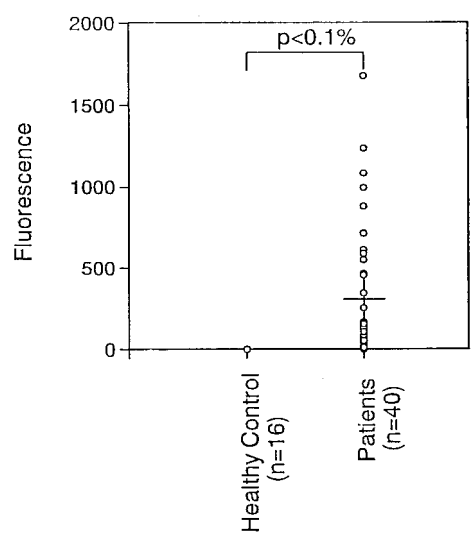

FIG. 2. Detection of specific Ig E to purified Cha o 2 in human sera. Sera from 40 allergic patients with pollinosis caused by *C. obtusa* (measured by AlaSTAT) and 16 non-allergic individuals were used, as described under Materials and Methods. Data are expressed as the means of duplicate samples, and were statistically analyzed by Aspin-Welch.

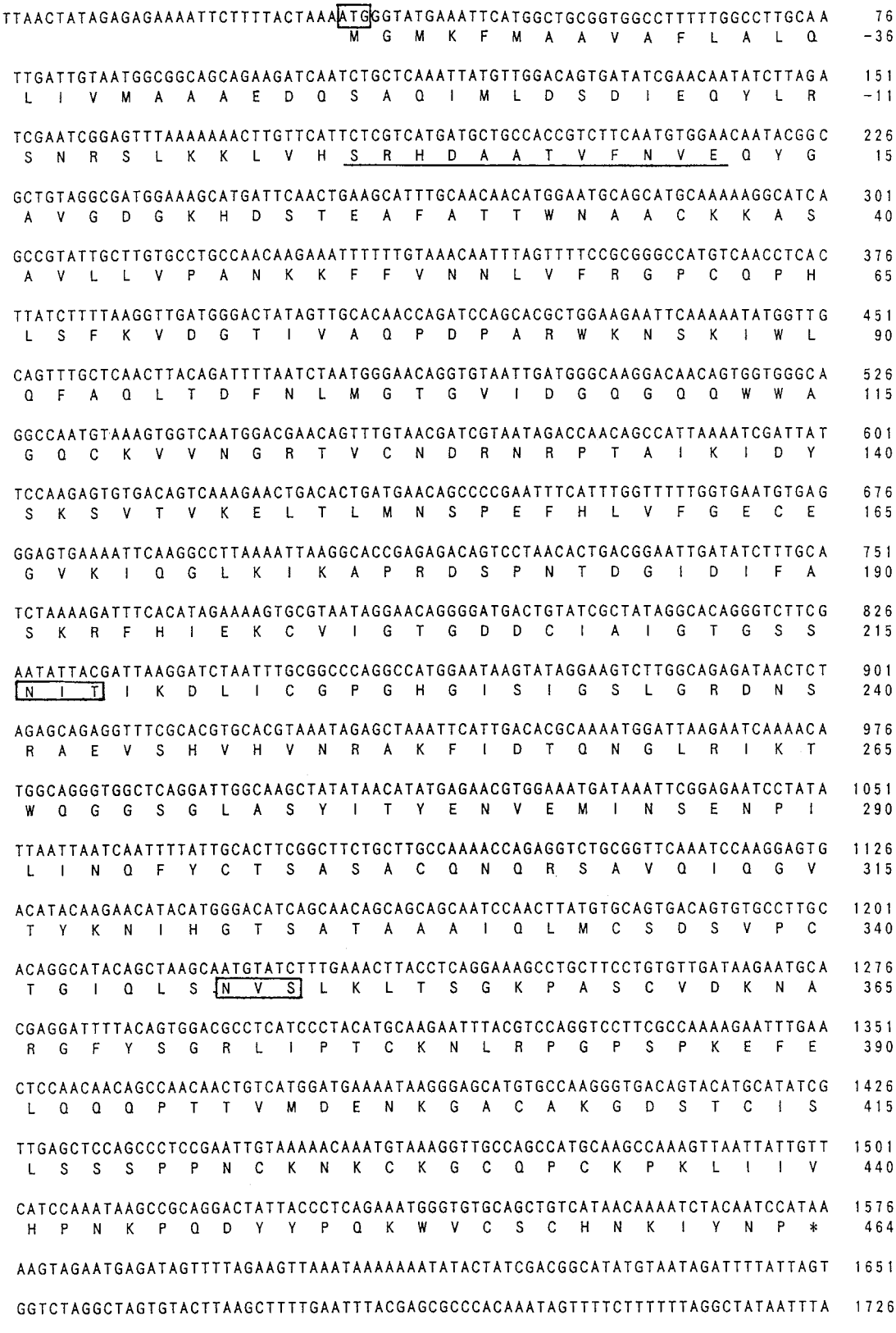

FIG. 3. Nucleotide and predicted amino acid sequences of a Cha o 2 cDNA clone. An ATG initiation codon is boxed and a TAA stop codon is indicated by an asterisk. Underlined amino acids were identified by amino acid sequencing of Cha o 2. The putative signal peptide is indicated by negative numbers. Potential glycosylation sites are boxed.

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Cha o 2 :  MGMKFMAAVA FLALQLIVMA AAEDQSAQIM LDSDIEQYLR SNRSLKKLVH SRHDAATVFN VEQYGAVGDG KHDST    75
           * **  *  * *** *** ** ********** ***  * *** ***** *  * *****   ** ** ******* *** *
Cry j 2 :  MAMKLIAPMA FLAMQLIIMA AAEDQSAQIM LDSVVEKYLR SNRSLRKVEH SRHDAINIFN VEKYGAVGDG KHDCT    75

Cha o 2 :  EAFATTWNAA CKKASAVLLV PANKKFFVNN LVFRGPCQPH LSFKVDGTIV AQPDPARWKN SKIWLQFAQL TDFNL   150
           *** * * ** **  ** *** *  *** *** * * ******   ***** *  *   ** ***   ****** * * * *
Cry j 2 :  EAFSTAWQAA CKNPSAMLLV PGSKKFVVNN LFFNGPCQPH FTFKVDGIIA AYQNPASWKN NRIWLQFAKL TGFTL   150

Cha o 2 :  MGTGVIDGQG QQWWAGQCKV VNGRTVCNDR NRPTAIKIDY SKSVTVKELT LMNSPEFHLV FGECEGVKIQ GLKIK   225
           ** ******* ******** **** **** ****** *  *       *  ********** ** ******  *  *
Cry j 2 :  MGKGVIDGQG KQWWAGQCKW VNGREICNDR DRPTAIKFDF STGLIIQGLK LMNSPEFHLV FGNCEGVKII GISIT   225

Cha o 2 :  APRDSPNTDG IDIFASKRFH IEKCVIGTGD DCIAIGTGSS NITIKDLICG PGHGISIGSL GRDNSRAEVS HVHVN   300
           ********** ******* **   *  ***** ** ******* ** * ***** ********** ** *******  ****
Cry j 2 :  APRDSPNTDG IDIFASKNFH LQKNTIGTGD DCVAIGTGSS NIVIEDLICG PGHGISIGSL GRENSRAEVS YVHVN   300

Cha o 2 :  RAKFIDTQNG LRIKTWQGGS GLASYITYEN VEMINSENPI LINQFYCTSA SACQNQRSAV QIQGVTYKNI HGTSA   375
            ********* ********** * ** * *** ********** ********** ********** *** ******  ****
Cry j 2 :  GAKFIDTQNG LRIKTWQGGS GMASHIIYEN VEMINSENPI LINQFYCTSA SACQNQRSAV QIQDVTYKNI RGTSA   375

Cha o 2 :  TAAAIQLMCS DSVPCTGIQL SNVSLKLTSG KPASCVDKNA RGFYSGRLIP TCKNLRPGPS PKEFELQQQP TTVMD   450
           ******* ** ** **  * * *  ******* * ***   **  *  **  **  **** *     **       *  ***
Cry j 2 :  TAAAIQLKCS DSMPCKDIKL SDISLKLTSG KIASCLNDNA NGYFSGHVIP ACKNLSPSAK RKESKSHKHP KTVMV   450

Cha o 2 :  ENKGACAKGD STCISLSSSP PNCKNKCKGC QPCKPKLIIV HPNKPQDYYP QKWVCSCHNK IYNP              514
           **  *  **   * * * * * *** *** **  *** ** ** *   ** *** * * **** * ** *
Cry j 2 :  ENMRAYDKGN RTRILLGSRP PNCTNKCHGC SPCKAKLVIV HRIMPQEYYP QRWICSCHGK IYHP              514
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FIG. 4. Sequence comparison of Cha o 2 and Cry j 2. The asterisks indicate an identical amino acid between them.

because the deduced amino acid sequence at positions 182 through 217 were completely identical to the N-terminal amino acid sequence of purified Cha o 2 protein (Fig. 3, underlined). To confirm this cDNA correspondence with Cha o 2 protein, Cha o 2 cDNA was cloned into the *E. coli* expression vector pQE9. Recombinant Cha o 2 was expressed and purified as a fusion protein with the histidine hexamer. As we expected, the anti-recombinant Cha o 2 was able to recognize both native Cry j 2 and Cha o 2 proteins (Fig. 1, lanes 5 and 6).

The N-terminal 50 amino acids coding the Cha o 2 cDNA might be a signal sequence because N-terminal amino acid sequence of mature protein did not contain this sequence. Therefore, native Cha o 2 was found to consist of 464 amino acids and the predicted molecular mass to be 50,490 Da. The pre-sequence of 50 amino acids is longer than typical signal peptides which consists of around 20–30 hydrophobic amino acids. While the first 22 amino acids were certainly hydrophobic, the residual 28 amino acids were rather hydrophilic (data not shown). This extended peptide region may have an additional unknown function. As we expected from the result of Zn^{2+}-chelating chromatography, Cha o 2 contains 12 histidine and 22 cysteine residues, which is much more than Cha o 1 (10 and 8, respectively).

Two potential N-linked glycosylation sites (Asn-X-Ser/Thr) were found at 271–273 and 402–404, but these positions were not conserved in Cry j 2.

Compared with the primary structure of Cry j 2, the length of the open reading frames and N-terminal extension peptides were almost identical. This might mean that the Cry j 2 and Cha o 2 genes have a common ancestor. The amino acid sequences of Cha o 2 and Cry j 2 displayed high homology (Fig. 4, 74.3% identical). Especially, the region 274–286, which contains a His-residue at position 278 and might be a part of the active site of polygalacturonase (10), which is completely conserved between some polygalacturonases (Tomato, Avocado and Maize) and Cry j 2 (4). These results indicate that the Cha o 2 protein might also belong to the family of polygalacturonases involved in the cell wall degradation associated with pollen tube penetration and expansion into pistils. The similarity between Cha o 2 and Cry j 2 is similar to that between Cha o 1 and Cry j 1, despite the evolutionary difference between cypress and cedar.

In the present study, we have demonstrated the purification and the molecular cloning of the cDNA of the Cha o 2 protein, the second major allergen of cypress pollen. The sequence information of Cha o 2 might be useful in the determination of T and B cell epitopes of

the allergen (11) and important for the development of immunotherapies against cypress pollinosis such as consensus allergens for cedar pollinosis (12).

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