

Molecular cloning of the mountain cedar (*Juniperus ashei*) pollen major allergen, Jun a 1

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Background: Cedar pollens cause allergic disease in diverse geographic areas. We have recently purified and characterized the major mountain cedar (*Juniperus ashei*) pollen allergen, Jun a 1.

Objective: A full-length complementary DNA for Jun a 1 was cloned and sequenced, and the recombinant protein was expressed.

Methods: Messenger RNA from mountain cedar pollen was purified and Jun a 1 sequences were established with use of reverse transcriptase-PCR and primers based on the N-terminal amino acid sequence of Jun a 1 and the homologous protein Cry j 1. Portions of the nucleotide sequence were confirmed by comparison with N-terminal amino acid sequencing of the intact tryptic fragments of the purified native protein. Recombinant Jun a 1 was cloned into pET 30, expressed in BL21, and purified by HPLC, and its allergenicity was analyzed by Western blotting with patient sera.

Results: Jun a 1 possesses a high level of amino acid sequence homology with Cha o 1 and Cry j 1, the major allergens of Japanese cypress and Japanese cedar. The amino acid sequence of a region with putative pectate lyase activity was identical to that of Cry j 1 and Cha o 1. Jun a 1 contained 2 potential N-glycosylation sites that were distinct from those found in Cry j 1. The IgE from patient sera bound recombinant Jun a 1 in Western blot analysis.

Conclusion: The high degree of homology of Jun a 1 with Cha o 1 and Cry j 1 may explain the cross-reactivity of conifer pollens. Differences in N-glycosylation suggest little overlap of glycopeptide epitopes. (J Allergy Clin Immunol 1999;104:613-7.)

Key words: Allergen, *Chamaecyparis obtusa*, *Cha o 1*, *Cry j 1*, *Cryptomeria japonica*, *Jun a 1*, *Juniperus ashei*, *Juniperus sabinoides*, mountain cedar, pollinosis

Abbreviations used

cDNA: Complementary DNA
mRNA: Messenger RNA
RT: Reverse transcriptase
TFA: Trifluoroacetic acid

Severe symptoms of rhinitis and asthma as a result of aeroallergens are major causes of morbidity, lost productivity, and increasing health care costs.¹ Hypersensitivity to cedar pollens is one of the most common causes of seasonal allergic disease.²⁻⁷ We recently purified and characterized the major allergen of mountain cedar (*Juniperus ashei*, Cupressaceae) pollen, Jun a 1 (see the accompanying article, Midoro-Horiuti et al, p •••). The molecular weight and N-terminal amino acid sequence of Jun a 1 was found to have a high degree of homology to Cry j 1 (the major allergen of *Cryptomeria japonica* pollen, Taxodiaceae) and Cha o 1 (the major allergen of *Chamaecyparis obtusa* pollen, Cupressaceae). Also, serum IgE from cedar allergic patients from Texas and Japan react with both Cry j 1 and Jun a 1 (see the accompanying article, Midoro-Horiuti et al, p 608).

Cry j 1 has been biochemically characterized and cloned.⁸ It consists of 353 amino acids and contains 2 N-glycosylation motifs. Cry j 1 has a region of significant sequence homology to pectate lyase and was shown to have enzyme activity.⁹

In this study we cloned and sequenced a complementary DNA (cDNA) of Jun a 1 and compared the sequence with the major allergens of other pollens.

MATERIAL AND METHODS

Mountain cedar pollen was obtained from Oklahoma and Arkansas, dried under vacuum for 48 hours, and stored in amber glass under nitrogen (Bayer).

Internal amino acid sequences of Jun a 1

Jun a 1 was purified as described (see the accompanying article, Midoro-Horiuti et al, p 608). Two milligrams of the HPLC-purified Jun a 1 was dissolved in 1 mL of 6 mol/L guanidine hydrochloride in 0.5 mol/L TRIS buffer containing 2 mmol/L EDTA (pH 8.2). Jun a 1 was reduced with use of 1.6 mg (about 50 molar excess over total disulfides) of dithiothreitol for 16 hours under nitrogen in the dark and alkylated with 1 mmol of iodoacetamide for 4 hours. The reduced and alkylated Jun a 1 was repurified by reversed-phase HPLC on a 218TP510 column equilibrated with solvent A (0.08%

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The nucleotide sequences of Jun a 1 appears in the GenBank sequence database under the accession numbers AF106662 and AF106663.

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trifluoroacetic acid [TFA]). Column elution was performed as described (see the accompanying article, Midoro-Horiuti et al, p 608). The reduced, alkylated, and lyophilized Jun a 1 was dissolved in 200 μ L of 2 mol/L urea in 0.1 mol/L TRIS buffer (pH 8.4) containing 0.01 mol/L calcium chloride. N-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (enzyme/substrate ratio = 1:50 [wt/wt], Promega) was added and incubated for 17 hours at 37°C. The trypsin digest was applied to an HPLC 218TP52 (Vydac) column equilibrated with solvent A. The elution was performed at a flow rate of 200 μ L/min with a gradient of solvent A and 0.08% TFA in acetonitrile (solvent B), 0% solvent A for 5 minutes, and a linear gradient from 0% to 45% of solvent B over 4 hours. The peptides were detected by measurement of ultraviolet absorbance at 215 nm.

In separate experiments purified Jun a 1 (200 μ g) was dissolved in 120 μ L of 0.1% SDS in 25 mmol/L ammonium bicarbonate (pH 7.8) and boiled for 5 minutes. V8 protease was added in a 1:3 ratio (wt/wt), twice during the 48-hour incubation at room temperature. The reaction was stopped by boiling for 5 minutes and the digested Jun a 1 was lyophilized.

A V8 peptide of Jun a 1 was purified by SDS-PAGE on 10% to 20% gradient gels. After the gel was stained with Coomassie blue, the 20-kD band was cut from the gel. The protein was electroeluted (electroeluter, Bio-Rad) for 17 hours in protein elution buffer (0.1% SDS, 25 mmol/L TRIS, 192 mmol/L glycine).

High-performance anion exchange chromatography with pulsed amperometric detection

Purified glycoprotein was subjected to 2 mol/L TFA hydrolysis for 4 hours at 100°C for detection of neutral and amino sugars. The monosaccharides were then analyzed by HPAEC-PAD with CarboPac PA 10 (4250 mm) column (Dionex, Sunnyvale, Calif).

Amino acid sequence and composition analysis

Jun a 1 or Jun a 1 peptides were hydrolyzed in 1-mL ampules with 0.5 mL of 5.7N hydrochloric acid in vacuo at 107°C for 24 hours. After hydrolysis the ampules were opened, dried in vacuo, and subjected to amino acid analysis. Before analysis the redissolved hydrolysates were filtered through 0.45-mm low-volume retention filters. Amino acid compositional analysis was carried out on a Beckman 6300 amino acid analyzer with use of a single-column protein-hydrolysat method. Primary structure analysis was determined with use of a Perkin Elmer Applied Procise microsequencer.

Messenger RNA isolation, cDNA synthesis, and reverse transcriptase-PCR amplification

Frozen pollen (100 mg) was ground in a mortar and pestle under liquid nitrogen. The resulting slurry was immediately vortexed for 12 minutes at room temperature with 800 mL of extraction buffer (10 mmol/L TRIS-H, pH 7.5, 1 mmol/L EDTA, 0.1 mol/L sodium chloride) to which 200 mL of glass beads (0.425–0.6 mm in diameter, Sigma, St Louis, Mo) were added. After centrifugation the supernatant was incubated with oligodeoxythymidine-cellulose (Pharmacia Biotech). Messenger RNA (mRNA) was eluted from the washed cellulose with elution buffer (10 mmol/L TRIS-H, pH 7.5, 1 mmol/L EDTA). The concentration and purity of mRNA was determined by its OD260/OD280 ratio. The mRNA was ethanol precipitated and first-strand cDNA was synthesized with oligodeoxythymidine (Gibco BRL, Gaithersburg, Md) and reverse transcriptase (RT) (Gibco BRL).

PCR was performed with use of sense (5'-CACAAAACA-

GAATGAAGCTC-3') and antisense primers (5'-TTGCAACATG-TACAAGTCC-3') on the basis of the Cry j 1 cDNA sequence.⁸ PCR amplification was performed for 28 cycles at 55°C annealing temperature. PCR products were analyzed by agarose gel electrophoresis and purified by the QIAquick gel extraction kit (Qiagen, Hilden, Germany).

Inverse PCR

Inverse PCR was used to clone 5' and 3' sequences.¹⁰ Second-strand cDNA was synthesized by the method of Okayama and Berg,¹¹ with use of *Escherichia coli* DNA ligase (New England BioLabs), *E. coli* DNA polymerase (New England BioLabs), and *E. coli* ribonuclease H (Promega, Madison, Wis) followed by blunt ending with T4 DNA polymerase (New England BioLabs). The mixture was extracted with phenol, chloroform, and isoamyl alcohol (24:24:1), followed by isopropanol precipitation. Double-stranded cDNA was circularized with T4 DNA ligase (Gibco BRL). Sense (5'-TATCGATGTTACGCTTGGCTC-3') and antisense (5'-TCATCTGCTCCCTGCCGTC-3') primers of Jun a 1 were synthesized on the basis of the sequences previously identified (see above).

5' and 3' end sequence analysis

As a second approach to obtain 5' or 3' unknown sequences, *EcoRI* adaptors (Gibco BRL) were ligated onto both ends of double-stranded cDNAs and the products ligated into Bluescript II SK⁻ vector (Stratagene). The Bluescript vector containing the cedar pollen cDNA was used as a template for PCRs in which T3 and T7 primers were paired with the original PCR primers to Jun a 1 sequence. The PCR products were purified, cloned, and analyzed as described above.

Southern blotting and DNA sequencing

PCR products were separated by electrophoresis in 1.5% agarose TBE gel and the DNA transferred to nylon membrane and hybridized with mountain cedar DNA (681 bp, initial PCR product) or an oligo probe as described previously.¹³ The cDNA inserts in pCR II were sequenced with a Perkin Elmer/Applied Biosystems model 373 A DNA sequencer with T7, SP6, or M13 as primers. Sequences were compared against the GenBank database using the Blast program in the Genetics Computer Group computer package (Madison, Wis). Potential T-cell epitopes were analyzed by T sites.¹⁴

Expression of recombinant Jun a 1 in *E. coli*

A full-length cDNA was generated by PCR amplification with engineered *NdeI* (5') and *EcoRI* (3') sites. Fidelity of the cDNA was confirmed by sequencing. The cDNA was inserted into the *NdeI* and *EcoRI* sites of the pET 30 plasmid vector and transfected into *E. coli* strain BL21. Transformants were selected by growth in kanamycin. Synthesis of recombinant Jun a 1 was induced in bacterial cultures with 2 mmol/L isopropyl β -D-thiogalactopyranoside. The bacterial cells were harvested by centrifugation, purified,¹⁵ and analyzed by SDS-PAGE and Western blotting as described above.

RESULTS

Nucleotide sequence and internal amino acid sequence of Jun a 1

The consensus DNA sequence obtained for Jun a 1 from 3 separate mRNA preparations as well as partial amino acid sequence analysis is shown in Fig 1. The full-length cDNA coding region spans 1101 nucleotides. The first 63 nucleotides containing ATG as the start codon

Jun a 1	DNPIDSCWRG	DSNWDQNRMK	LADCAVGFGS	STMGGKGGDF	YTVTS*TDDNP	50
Cha o 1		.A.....		.A.....A.	...S*S..D.	
Cry j 1		...A.....		L	...N*S..D.	
Amb a 1	L.I..K....	KAD.EN..QA	Q..AK	G.Y...W..V	NL..DV	
Amb 2 2	H.I..K...C	KPD.AE..QA	.G...Q...K	A.H...W.DI	.M...DQ..DV	
Jun a 1	VNPTPTLRY	GATREKALWI	IFSQNMNIKL	KMPLYVAGHK	TIDGRGADVH	100
Cha o 1	...A.....	RS...	...K.L....	N....I..N.	E..	
Cry j 1	...A.....	DRP...	...G.....	...M.I..Y.	.F....QV.	
Amb a 1	A..KE....F	A.AQNRP...	..K.DMVIN.	NQE.V.NSD.	VK.E	
Amb a 2	...KE....F	...QDRP...	..QRDMI.Y.	QQEMVVTSD.	K.E	
Jun a 1	LGNNGPCLFM	RKVSHVILHS	LHIGCNTSV	LGDVLVSESI	GVEPVHAQDG	150
Cha o 1	I.....	.T.....G	.N.....	S.N..I..AS	..V.....	
Cry j 1	I.....V.I	KR..N..I..	.YLY..S...	..N..IN..F	P...	
Amb a 1	II...LT.*.	N*.KNI.I.N	IN..DVKVLP	G.MIK*.NDG	PPILRQ.S..	
Amb a 2	LVY..IT.*.	N*.KN..I.N	ID..DVRVLP	G.RIK*.NGG	PAI.R.QS..	
Jun a 1	DAITMRNVTN	AWIDHNSLSD	CSDGLIDVTL	GSTGITISNN	HFFNHHKVML	200
Cha o 1	D	V.....	S...V....	A...V....		
Cry j 1	..L.L.TA..	I.....F.N	S...V....	T..V....	L.....S	
Amb a 1	.T.NVAGSSQ	I....C...K	SF.....	...H....C	K.TQQS.AI.	
Amb a 2	...HVTGSSD	I....CT..K	SF.....NW	...V....	K.TH.E.AV.	
Jun a 1	LGHDDTYDDD	KSMKVTVAFN	QFGPNAGQRM	PRARYGLVHV	ANNNDPWN	250
Cha o 1	...S.I.S..			I..	S.	
Cry j 1	...A.S..		C...		T.	
Amb a 1	..A...HVQ.	.G.LA....	M.TD.VD...	..C.F.FFQ.	V.....R.GT	
Amb a 2	..A...HFQ.	LK.H..L.Y.	I.TNTVHE..	..C.F.FFQI	V..F..R.DK	
Jun a 1	YAIGSSNPT	ILSEGNSTFA	PSESYKKEVT	KRIGCESPSA	CANWVWRSTR	300
Cha o 1			.ND.D....	R.V.....T	Q	
Cry j 1			.NE...Q..	I...KTS.S	.S...Q..Q	
Amb a 1	A..	..CQ..R.L.	.DDQI..N.L	ART.TGAES	M*.N...DK	
Amb a 2		...Q..K.V.	.DFIY..N.C	L.T.AQE.EW	M*T.N..TQN	
Jun a 1	DAFINGAYFV	SSGKTEETNI	YNSNEAFKVE	NGNAAPQLTK	NAGVVT*	346
Cha o 1	.S.N.....	...N.G...	..N.....	..S...H..Q	...L.CILS	
Cry j 1	.V.Y.....	...Y.GG..	.TKK...N..	...T.H..Q	...L.CSL	
Amb a 1	.LLE...I..	T..SDPVLTP	VQ.AGMIPA.	P.E..IK..S	S...SCRPG	
Amb a 2	.VLE...I..	A..SDPVLTA	EQ.AGMMQA.	P.DMV....M	CSPG	
Jun a 1		Identities				
Cha o 1	KPCS	85.5%				
Cry j 1	KRC*	78.9%				
Amb a 1	APC*	48.8%				
Amb a 2	APC*	48.3%				

FIG 1. Homology analysis. The amino acid sequence of Jun a 1 is shown compared with those of Cha o 1, Cry j 1 (clone pCC12-2), Amb a 1, and Amb a 2. Jun a 1 had 21 amino acid sequence, MASPCLIADVFLCAIVSCYS (putative leader sequence), followed by mature protein sequence. The putative pectate lyase motif is outlined by a box. Predicted T-cell epitopes of Jun a 1 and reported epitopes of Cry j 1 are underlined (as predicted by T-sites¹⁴). Some of the T-cell epitopes in Cry j 1 are underlined twice because these were reported by 2 authors.¹⁵⁻¹⁷

likely represent a signal peptide because these amino acids are not found in the purified protein. The sequence of the N-terminal amino acids was identical to that inferred by the nucleotide sequence. A TAA stop codon was found at position 1039, which would result in a mature protein of 346 amino acids. The 5' and 3' untranslated regions are not shown. The cDNA contained a sequence (Arg²²⁹MetProArgAlaArgTyrGlyLeuValHisValAlaAsnAsnAsnTyr²⁴⁵) that was identical to the pectate lyase active site of Cry j 1. Two potential N-glycosylation sites were present at Asn¹²⁷ and Asn¹⁵⁷. Several independently isolated cDNA clones identified a polymorphism at nucleotide 621, T (AF106662) or C (AF106663). Both sequences encode for Tyr²⁰⁷.

Trypsin and V8 protease digestion of Jun a 1

To confirm the internal amino acid sequence of Jun a 1 and to identify glycosylation sites, we determined the amino acid sequences of several tryptic and V8 protease peptides. Peptides derived from the tryptic digest of Jun a 1 separated into 12 major peaks by HPLC. Carbohydrate analysis indicated that 3 of these were glycosylated. The internal tryptic peptide sequences are Gly¹⁰ Asp Ser Asn Trp Asp Gln Asn, Leu²¹ Ala Asp Cys Ala Val Gly Phe Gly, Gly³⁷ Gly Asp Phe Tyr Thr Val Thr, Thr⁴² Val Thr Ser Thr Asp Asp Asn Pro Val Asn Pro Thr Pro Gly Thr Leu Arg, Ala⁶⁷ Leu Trp Ile Ile Phe Ser, Gly⁹⁶ Ala Asp Val His Leu Gly Asn, Val¹¹³ Ser His Val Ile Leu His,

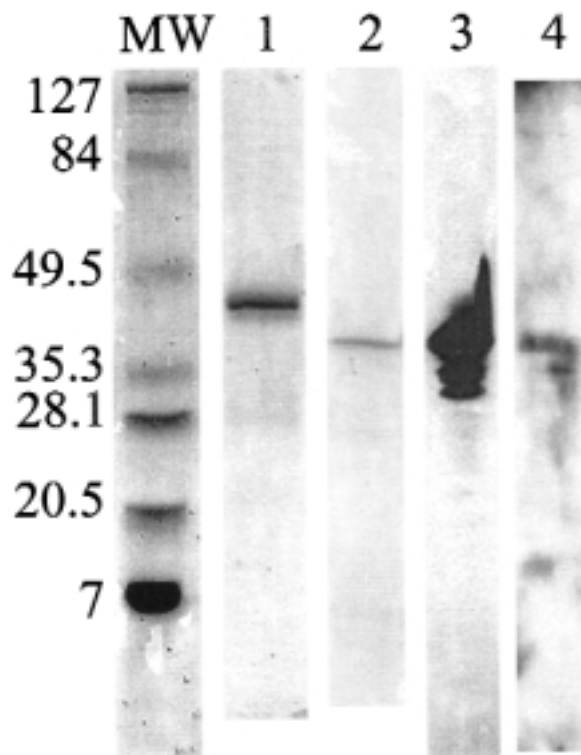


FIG 2. Electrophoretic pattern of native Jun a 1 and recombinant Jun a 1. Native (lane 1) and recombinant Jun a 1 (lanes 2-4) were analyzed by SDS-PAGE and Western blotting. Detection was performed with Coomassie blue stain (lanes 1 and 2), anti-Cry j 1 (lane 3), and antihuman IgE from pooled patients sera (lane 4).

Tyr²⁴⁵ Gly Leu Val His Val Ala Asn, and Asp³⁰¹ Ala Phe Ile.

A 20-kD fragment and several smaller fragments were detected in the V8 protease digest by SDS-PAGE. The 20-kD fragment was electroeluted and its N-terminal amino acid sequence determined (Ser¹³⁹ Ile Gly Val Glu). The previously reported T-cell epitopes of Cry j 1¹⁶⁻¹⁸ and potential T-cell epitopes analyzed by T sites¹⁴ are shown in Fig 1.

Homology comparison of the amino acid sequence of Jun a 1

The amino acid sequence of Jun a 1 is compared with those of Cry j 1, Cha o 1, Amb a 1, and Amb a 2 (the major allergens of ragweed, *Ambrosia artemisiifolia*, Compositae) in Fig 1. Jun a 1 had a high degree of sequence identity to Cha o 1 (85.5%) and Cry j 1 (78.9%) but also had a significant degree of sequence identity to Amb a 1 (48.8%) and Amb a 2 (48.3%).

Recombinant Jun a 1

Lysates of *E coli* transformed with plasmid DNA encoding Jun a 1 contained a 40-kD protein that was positive in Western blotting with anti-Cry j 1 polyclonal antibody (Kowa, Tsukuba, Japan)¹⁹ and IgE from cedar-hypersensitive patients (Fig 2).

DISCUSSION

Cedar hypersensitivity is one of the most frequent causes of seasonal allergic disease. We cloned and sequenced the major allergen of mountain cedar, Jun a 1, which is homologous to the major allergens of Japanese cypress and Japanese cedar. The amino acid sequence of the mature Jun a 1 protein contained 346 residues, making it similar in length to Cry j 1 and Cha o 1. Portions of the Jun a 1 protein sequence were determined by amino acid sequence analysis of the N-terminus and 4 internal peptides produced by trypsin and V8 protease digestion of the native Jun a 1 glycoprotein. The inferred and deduced amino acid sequences of Jun a 1 were identical at all residues and had a high degree of homology to those of Cha o 1 and Cry j 1.^{8,20} This is consistent with previous reports that the pollens of Taxodiaceae and Cupressaceae families may have allergenic cross-reactivity and induce cross-hypersensitivity.^{9,21}

The major IgE epitope of Cry j 1 has been reported to be a glycopeptide at asparagine 333.²² This site represents 1 of 2 demonstrated N-glycosylation sites (residues 170 and 333). Three other potential N-glycosylation sites (residues 137, 258, and 272) were found in the Cry j 1 cDNA sequence.⁸ The cDNA sequence of Jun a 1 demonstrated 3 N-glycosylation motifs. On the basis of carbohydrate analysis of proteolytic fragments, it appears that all 3 of these sites are glycosylated on at least some of the Jun a 1 molecules. Interestingly, neither of the proved and only one of the potential N-glycosylation sites of Cry j 1 (Asn²⁵⁸) were conserved in Jun a 1. Conversely, 2 of the potential N-glycosylation sites (residues 52 and 157) in Jun a 1 were not present in Cry j 1. Thus only 1 of 7 potential N-glycosylation sites is shared by Cry j 1 and Jun a 1 (Asn²⁵⁸). Therefore much of the cross-reactivity between Cry j 1 and Jun a 1 may not be due to shared glycoprotein epitopes but rather to either pure carbohydrate moieties or to regions of conserved amino acid sequence.

Cry j 1 has been shown to have pectate lyase activity.⁹ The amino acid sequence of Cry j 1 that imparts this activity is completely conserved in Jun a 1, suggesting that Jun a 1 may have pectate lyase activity. The substrates of pectate lyase are pectic substances found in the cell walls of plant tissues.²³ The physiologic role of Jun a 1 in pollen may be to facilitate pollen tube growth during pollination by degrading the pistil of female flowers, as speculated for Cry j 1.²³ The allergenic cross-reactivity between different families of plant allergens might be due to a conserved pectate lyase motif. It may therefore be important to investigate the IgE response to this region of conserved structure. Pectate lyase may also hydrolyze mammalian structures, such as the glycocalyx, which is on the luminal surface of epithelial cells. Thus, the pectate lyase activity of pollen glycoproteins such as Jun a 1 may facilitate penetration through the cellular glycocalyx, thereby enhancing the allergenicity of that and other pollen proteins.

Jun a 1 has a high degree of homology to some of the

T-cell epitopes of Cry j 1 sequences. Ikagawa et al¹⁶ reported that p335-346 represented a major epitope of Cry j 1. The substitution of Thr³³⁹ to valine, glycine, or glutamine altered the cytokine production by stimulated T cells remarkably. Threonine 339 was conserved in Jun a 1. These conserved regions of Jun a 1 and Cry j 1 may be important to the recognition of the conifer pollens by T cells, but cross-stimulation assays have not been performed. Jun a 1 also has about 50% sequence identity to ragweed allergens (Amb a 1 and Amb a 2), but cross-reactivity between these groups of allergens has not been reported. It is possible that major epitopes exist on non-homologous portions of these allergens. Interestingly, significant differences exist within the pectate lyase motif of ragweed and cedar allergens.

In conclusion, we have cloned a major allergen from mountain cedar pollen. The high degree of sequence identity between this allergen and those of other conifers and even ragweed may explain some common IgE epitopes among plants. A more complete characterization of the biologic and immunologic roles of shared structures among these allergens may help to explain their allergenicity and suggest new approaches for preventing and treating cedar pollinosis.

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