

Cupressaceae Pollinosis: Identification, Purification and Cloning of Relevant Allergens

Gabriella Di Felice Bianca Barletta Raffaella Tinghino Carlo Pini

Department of Immunology, Istituto Superiore di Sanità, Rome, Italy

Key Words

Cupressaceae · Pollinosis · Cross-reactivity · Purified allergens · Recombinant allergens

Abstract

Allergy to Cupressaceae pollen is a worldwide pollinosis caused by several species. Pollen extracts prepared from allergenic species belonging to this family are characterised by low protein and high carbohydrate content. The allergenic components represented in the pollen extracts from different species of the Cupressaceae family show high levels of cross-reactivity when probed with human IgE from allergic subjects and share a number of common epitopes also identified by polyclonal rabbit antisera and monoclonal antibodies. A close relationship has also been described with the Taxodiaceae and Podocarpaceae families. Although both proteic and carbohydrate epitopes appear to be involved in IgE recognition and allergenic cross-reactivity, a large portion of the IgE reactivity of Cupressaceae-allergic patients seems to be associated with sugar moieties present on the relevant allergenic molecules. From this point of view, Cupressaceae/Taxodiaceae allergens constitute a particularly useful model to study IgE cross-reactivity, as they have been shown to display different levels of homology. Moreover, the availability of the purified allergens, together with their recombinant counterparts, may shed light on the actual role played by carbohydrate in allergic sensitisation, IgE recognition and allergenic cross-reactivity.

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Introduction

Species of the Cupressaceae family are a very important cause of respiratory allergies in various geographic areas, including North America (Texas and south-western states) [1, 2], South Africa [3], Australia [4] and Mediterranean countries [5], such as France [6], Italy [7, 8], Spain [9] and Israel [10]. In Japan, the major pollen allergen source is the Japanese cedar (*Cryptomeria japonica*), a member of the closely related family of Taxodiaceae [11–13], and another important aeroallergen is represented by Japanese cypress (*Chamaecyparis obtusa*) [14].

Incidence data for 'cypress/cedar/juniper' pollinosis have indicated an increasing trend in the last 10 years, in some areas reaching about 30% of all the cases of pollinosis [9, 15–17]. The reasons for such an increase could lie in several concomitant causes, including: (1) the increased exposure to Cupressaceae species, due to reforestation programs, gardening, wind barriers and widespread landscaping using nonindigenous species (for example, in Italy [7]); (2) a previous potential underestimation of the real prevalence of cypress allergy, related to the low potency of diagnostic extracts formerly available [18], or to the symptom type and period, often mimicking those of generic winter disease, which impede a correct diagnosis of pollinosis [19], and (3) the potential adjuvant effect of increasing air pollution [20].

The allergenic species belonging to the Cupressaceae, Taxodiaceae and Podocarpaceae families share several distinctive characteristics, such as the isolated pollen sea-

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Correspondence to: Gabriella Di Felice
Department of Immunology, Istituto Superiore di Sanità
Viale Regina Elena 299
I–00161 Rome (Italy)
Tel. +39 06 49902871, Fax +39 06 49387115, E-Mail difelice@iss.it

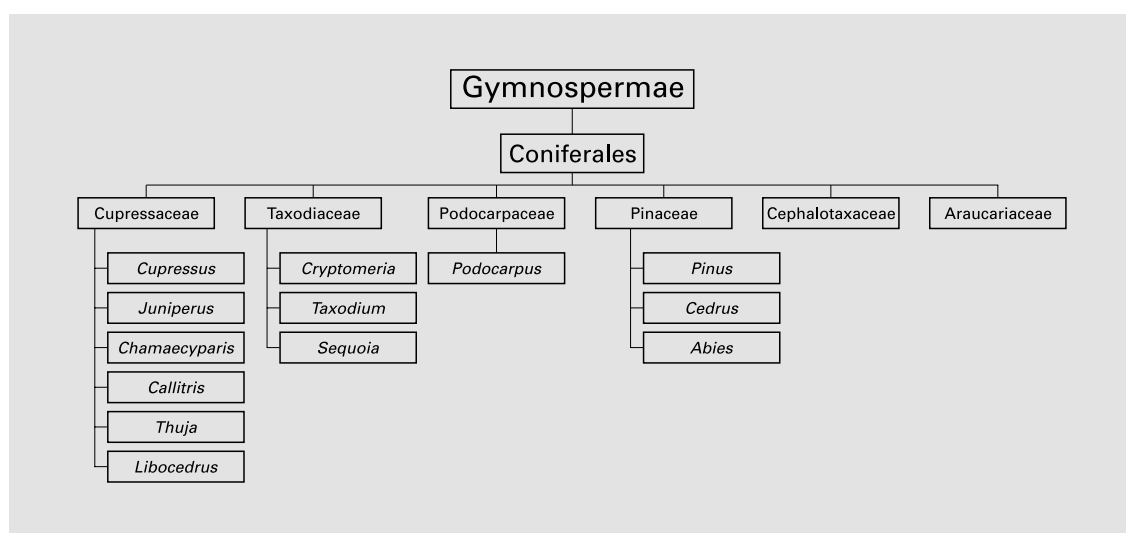


Fig. 1. Taxonomy of Cupressaceae species. Allergenic species are found in four of the six Coniferales families (Cupressaceae, Taxodiaceae, Podocarpaceae and Pinaceae). Among these, the Cupressaceae, Taxodiaceae and Podocarpaceae families exhibit a high level of allergenic cross-reactivity.

son in winter or early spring (whereas many other allergenic species flower only later in spring/summer), the diffusion in widespread areas with different geographic and climatic conditions, the low protein and high carbohydrate content in the pollen extract and the high level of allergenic cross-reactivity.

This article will review the main features of Cupressaceae pollinosis, as well as the immunochemical and immunological features of the allergenic molecules identified, purified and cloned in the pollen extracts from these species.

Cupressaceae Pollinosis: Taxonomic, Clinical and Immunological Data

The Coniferales order, belonging to the Gymnospermae class, contains six families, four of which include species that are of interest to the allergist (fig. 1): Cupressaceae, Taxodiaceae, Podocarpaceae and Pinaceae. The first three families show high levels of cross-reactivity and the Pinaceae family is more distantly related [21]. The number of pollen species from Cupressaceae and closely related families (Taxodiaceae and Podocarpaceae), which are documented as respiratory sensitizers (table 1), is growing, supporting an important role of these families in late autumn, winter and early spring pollinosis. The gen-

era for which a significant clinical incidence has been reported are the following: *Cupressus*, *Juniperus*, *Chamaecyparis*, *Callitris*, *Thuja*, *Libocedrus* (Cupressaceae); *Cryptomeria*, *Taxodium* (Taxodiaceae), and *Podocarpus* (Podocarpaceae) [1, 2, 8, 10–14, 18, 22–25].

Analysis of the clinical features of cypress-sensitized patients reveals some peculiarities. The prevalent clinical symptom is rhinitis, often associated with conjunctivitis, whereas the incidence of asthma is generally lower as compared with patients sensitized to other allergenic sources [16, 26–28]. Patients monosensitized to Cupressaceae and those allergic also to multiple pollen species appear to be heterogeneous in terms of immune reactivity [26, 29]. Patients allergic only to cypress pollen have normal or subnormal total serum IgE levels and lower specific IgE, as compared with polysensitized patients [26, 29]. Moreover, it has been reported that they develop symptoms at an older age than polysensitized patients and in general than most pollen-allergic patients in the same geographical area [26, 29]. Despite the high level of allergenic cross-reactivity between Cupressaceae and Taxodiaceae species, the clinical features described above are not shared with Japanese cedar pollinosis in the Japanese population.

Table 1. Species of allergenic interest belonging to the Cupressaceae, Taxodiaceae and Podocarpaceae families

Family	Genus	Species	Common names
Cupressaceae	<i>Cupressus</i>	<i>sempervirens</i>	Italian cypress
Cupressaceae	<i>Cupressus</i>	<i>arizonica</i>	Arizona cypress, blue cypress
Cupressaceae	<i>Cupressus</i>	<i>glabra</i>	
Cupressaceae	<i>Cupressus</i>	<i>lusitanica</i>	
Cupressaceae	<i>Cupressus</i>	<i>macrocarpa</i>	Monterey cypress
Cupressaceae	<i>Juniperus</i>	<i>ashei</i>	mountain cedar
Cupressaceae	<i>Juniperus</i>	<i>virginiana</i>	eastern red cedar
Cupressaceae	<i>Juniperus</i>	<i>communis</i>	common juniper
Cupressaceae	<i>Juniperus</i>	<i>oxycedrus</i>	prickly juniper
Cupressaceae	<i>Juniperus</i>	<i>pinchotii</i>	Pinchot juniper
Cupressaceae	<i>Juniperus</i>	<i>osteosperma</i>	Utah juniper
Cupressaceae	<i>Juniperus</i>	<i>monosperma</i>	one-seed juniper
Cupressaceae	<i>Juniperus</i>	<i>rigida</i>	Japanese juniper
Cupressaceae	<i>Chamaecyparis</i>	<i>obtusa</i>	Japanese cypress (Hinoki)
Cupressaceae	<i>Chamaecyparis</i>	<i>lawsoniana</i>	Port Orford cedar
Cupressaceae	<i>Libocedrus</i>	<i>decurrens</i>	incense cedar
Cupressaceae	<i>Callitris</i>	<i>glaucophylla</i>	white cypress pine
Cupressaceae	<i>Callitris</i>	<i>verrucosa</i>	
Cupressaceae	<i>Thuja</i>	<i>orientalis</i>	
Cupressaceae	<i>Thuja</i>	<i>occidentalis</i>	eastern white cedar
Cupressaceae	<i>Thuja</i>	<i>plicata</i>	western red cedar
Taxodiaceae	<i>Cryptomeria</i>	<i>japonica</i>	Japanese cedar (Sugi)
Taxodiaceae	<i>Taxodium</i>	<i>distichum</i>	bald cypress
Taxodiaceae	<i>Sequoia</i>	<i>sempervirens</i>	California redwood
Podocarpaceae	<i>Podocarpus</i>	<i>gracilior</i>	

Identification, Characterisation and Purification of Cupressaceae Allergens

Analysis of the allergenic component in pollen extracts from Cupressaceae species has proved difficult, due to the low protein content of aqueous extracts. Low protein and high carbohydrate content is in fact a characteristic of pollen extracts from several species [8, 18, 30], which has for a long time hindered the availability of standardised extracts for the diagnosis and immunotherapy of cypress pollinosis. As a first step in achieving this aim, efforts were made to establish the optimal conditions for pollen extraction and to provide a qualitative definition of the relevant allergenic components in the extracts. The results obtained in this field by the work of different groups on several Cupressaceae and Taxodiaceae species allowed the preparation of good-quality allergenic extracts as well as their biochemical characterisation [8, 11, 18, 22, 24, 30, 31], and constituted the basis for the study of cross-reactivities between species and families. Taken together, these results led to a significant improvement in the available tools for the diagnosis of Cupressaceae pollinosis,

and consequently in the sensitivity and accuracy of the diagnosis itself [15].

Analysis of pollen extracts in IgE immunoblotting identified the major allergenic component, predominant both in terms of the intensity and frequency of recognition by IgE from allergic patients, in a glycoprotein with a molecular weight of 41,000–45,000 D [11, 18, 30, 32]. The isolation and characterisation of this relevant allergen were first achieved in *Juniperus ashei* and *C. japonica* pollen extracts. A biologically active fraction of about 50,000 D was obtained by gel chromatography fractionation of the pollen extract from *J. ashei*, and the N-terminal amino acid sequence was determined [32]. Yasueda et al. [11] reported the isolation of the major allergen from Japanese cedar (*C. japonica*) pollen extract, its biochemical characterisation and in vivo and in vitro assay of its allergenic activity. This allergen was firstly designated as antigen SBP (Sugi basic protein) and described as a heat-sensitive basic glycoprotein of approximately 40,000 D in molecular weight. Purification of this allergen was accomplished by ammonium sulphate precipitation, followed by ion exchange chromatography and gel chromatography.

By immunodiffusion analysis, direct skin testing and RAST inhibition, this purified molecule was demonstrated to be the major allergen of Japanese cedar pollen. Further purification of this allergen, renamed Cry j 1 according to the rules of the Allergen Nomenclature Committee, was performed by Taniai et al. [33] in order to analyse the N-terminal amino acid sequence. Five sub-fractions of Cry j 1 were separated and sequenced, showing similar patterns of human IgE reactivity, N-terminal sequencing and peptide mapping, with some differences in molecular weight ascribed to the existence of N-linked carbohydrate chains. Interestingly, the authors found that the N-terminal amino acid sequence of Cry j 1 was identical to the N-terminal 5 amino acid sequence of *J. ashei* reported by Gross et al. [32]. During the analysis of the partial amino acid sequence of purified Cry j 1, a peptide fragment was found which exhibited significant homology to some pectate lyase isozymes secreted by plant pathogenic bacteria [34]. Evaluation of enzymatic activity showed that whole purified Cry j 1 had a specific pectate lyase activity of 350 U/mg of protein. The enzymatic function had an absolute Ca^{2+} ion requirement [34]. These findings allowed a better elucidation of the physiological role of Cry j 1 in the pollen grain, since the pectic substances working as a substrate of pectate lyase are found in the cell wall of all plant tissues. Therefore, taking into account that this allergenic molecule is produced during the mature stages of pollen development and is localised on the exine layer of the pollen grain [35], the physiological role of Cry j 1 may be to facilitate pollen tube growth during pollination by degrading female flowers. The Cry j 1 peptide fragment shows significant homology with the major allergens from *Ambrosia artemisiifolia* pollen (Amb a 1, 65% identity [36], and Amb a 2, 53% identity [37]), thus suggesting that pectate lyase activity could also be exerted by these allergens. The fact that three major allergens from completely different plants have an homologous peptide region suggested that this region may play an important role in the immune response to these allergens [34]. In support of this possibility, evidence has been presented that the peculiar folding unit of this class of enzymes, called the parallel β helix, could provide an ideal template for antigen presentation [38].

The second major allergen from Japanese cedar pollen, Cry j 2, was isolated and characterised by Sakaguchi et al. [39]. Cry j 2, a protein of 37,000 D in molecular weight with an N-terminal amino acid sequence completely different from that of Cry j 1, did not show any allergenic cross-reactivity with Cry j 1, as demonstrated by IgE

ELISA inhibition studies. Studies with a panel of 23 monoclonal antibodies (mAbs) raised against Cry j 1 and Cry j 2 [40, 41] led to the fine analysis of B cell epitopes displayed by the two allergens, confirming the absence of cross-reactive determinants on the two molecules. From a functional point of view, Cry j 2 has been classified as a polymethylgalacturonase, although existence of this activity has not been conclusively proven [42]. Therefore, it is conceivable that both Cry j 1 and Cry j 2 may act together to facilitate pollen tube growth during pollination with their enzymatic properties.

At least four antigenic determinant regions have been identified in the Cry j 1 allergen, by classifying into four groups the fine specificity of the mAb panel, as determined in direct binding assays and cross-inhibition studies. Two of the four epitopes seemed to be conformational, whereas the remaining two were of a sequential structure. The blocking of human IgE binding by some of the mAbs suggested the relevance of the mAb-defined epitopic regions in the human IgE recognition and allergic response. Epitope mapping of the Cry j 1 molecule using both mAbs and human IgE allowed the identification of a glycopeptide, the glycosylation of which might be required for the IgE binding of the peptide [43]. This finding opened the way to a series of studies aimed at the characterisation of the oligosaccharide moiety of the Taxodiaceae and Cupressaceae major allergens, and in general the role played by carbohydrate residues in IgE recognition and IgE cross-reactivity.

The initial studies on the major oligosaccharides of Cry j 1 revealed a series of compounds carrying biantennary glucosamine branches linked to α -mannosyl residues of the common core unit, $\text{Man}\alpha 1-6(\text{Man}\alpha 1-3)(\text{Xyl}\beta 1-2)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4(\text{Fuc}\alpha 1-3)\text{GlcNAc}$ [44], which are widely represented in plant glycoproteins. These carbohydrate structures were found to be antigenic in rabbit [45], whereas their contribution to the reactivity of specific IgE to Cry j 1 appeared to be small or only conformational, in that they may contribute to the formation and stability of active conformational epitopes [44].

As more allergens from several Cupressaceae species became available in a purified form, the close relationship between the corresponding major allergenic molecules of the species belonging to this family was confirmed. Cha o 1 and Cha o 2 from *C. obtusa*, the second pollen responsible for respiratory allergies in Japan [14, 46], have been purified by chromatographic methods and characterised [47–49]. The major allergen from mountain cedar (*J. ashei*) pollen, Jun a 1, was isolated from salt-precipitated pollen extracts by affinity chromatography on a conca-

navalin A column or by HPLC reversed-phase chromatography on a hydrophobic column [50].

By this separation procedure, another allergenic protein has been purified [51], named Jun a 3 since it shows no homology with the previously identified Cupressaceae allergens. Jun a 3, showing a molecular weight of about 30,000 D, was found to be homologous to the thaumatin-like pathogenesis-related group 5 (PR-5) plant proteins, and it was the first member of this group of proteins identified as a pollen allergen. Plant PR proteins appear to be upregulated by environmental stress conditions, including some pollutants, and, in fact, Jun a 3 is variably expressed in different lots of pollens collected from different regions and in different years. On this basis, the authors speculated that environmental factors may also indirectly influence allergic diseases through the altered pollen expression of PR proteins [51].

Ad hoc pollen extraction procedures followed by chromatographic separations were set up for the purification of Jun a 2 [52] and Cup a 1 [53]. MALDI-TOF mass spectrometry analysis of purified Cup a 1 indicated the presence in different amounts of three N-linked oligosaccharide structures, all of which carry both xylose and core α 1,3-fucose. Interestingly, a significant fraction of Cup a 1 N-glycans carry the Lewis^a epitope Gal β 1,3(Fuc α 1,4)-GlcNAc on one arm. These structures are similar to those described in the characterisation of the Cry j 1 glycan moiety.

Molecular Cloning

In recent years, interest has been focused on recombinant DNA technology as a powerful tool to produce and characterise allergenic molecules [54]. The availability of recombinant allergenic molecules may contribute to the standardisation of allergens used for the diagnosis and immunotherapy of allergic disease [55]. Moreover, knowledge of the complete amino acid sequence facilitates the determination of T and B cell epitopes along the allergenic molecule, thus suggesting new approaches for preventing and treating allergic disease. Finally, the evaluation of sequence homology provides information on IgE-binding structures shared among close or distant species and on their biological and immunological functions, contributing from a molecular point of view to the study of allergenic cross-reactivity.

In this respect, an important point to consider is the correct evaluation of the reactivity of the recombinant allergen with human IgE. This assessment is the basis for

their use both as standards for the evaluation of native allergen and allergenic extracts and as tools for designing new diagnostic and therapeutic approaches. The use of different expression systems (bacteria, yeasts, plants) can affect IgE recognition, as well as other biological activity, according to their different capacity to introduce post-translational modifications, for example glycosylation, in the recombinant molecule. This aspect should be carefully considered especially for glycoprotein allergens, in which the carbohydrate moiety is directly or indirectly involved in IgE recognition, as suggested for Cupressaceae major allergens. The evaluation protocol should include validated methods, including in vitro IgE binding, histamine release assay, testing in animal models and, when applicable, direct skin testing in allergic patients [56].

Among the Taxodiaceae/Cupressaceae species, several allergenic molecules from different species have been cloned and characterised (table 2).

The major allergen Cry j 1 was first cloned by the PCR amplification technique [57] with primers deduced from the sequence of the N-terminal and internal peptides, obtained from the biochemically purified molecules. The deduced amino acid sequence of Cry j 1 consisted of a 374-residue polypeptide [57], which contained a 21-residue signal peptide and a 353-residue mature protein. The molecular weight of mature Cry j 1 was calculated to be 38,500 D, compared to the molecular weight of 41,000–46,000 D found for the native protein. Since five possible N-linked glycosylation sites (with the characteristic Asn-X-Ser/Thr motif) were found along the sequence, the difference has been ascribed to the glycosylation of the molecule. The study of nucleotide differences in different clones resulting in amino acid exchanges indicated that the genes coding for Cry j 1 are polymorphic. The comparison analysis performed with the complete sequence confirmed the presence of about 45% homology with Amb a 1 and Amb a 2 allergens, and 24–38% homology with some pectate lyase enzymes from different origins.

Due to the high degree of homology between purified Cha o 1 and Cry j 1, the major allergen from *C. obtusa* was cloned by using a Cry j 1 clone as a probe to screen the cDNA library constructed from *C. obtusa* pollen [47]. The longest clone obtained was found to code for a putative 21-residue signal peptide and a 354-residue mature protein with a derived molecular weight of 38,000 D. The deduced amino acid sequence of Cha o 1 showed 79–80% identity with Cry j 1 and 46–49% identity with Amb a 1 and Amb a 2 families. On the basis of the high degree of homology with Cry j 1, a putative pectate lyase enzyme activity was also suggested for Cha o 1 (table 3).

Table 2. Recombinant allergens from Cupressaceae and Taxodiaceae species

Allergen name	Molecular weight, D	Species	Biological function	Cross-reactivity	Reference
Cry j 1	38,500	<i>Cryptomeria japonica</i>	pectate lyase	within Cupressaceae/Taxodiaceae; Amb a 1, Amb a 2; pectate lyase enzyme family	57
Cry j 2	42,000	<i>Cryptomeria japonica</i>	polygalacturonase	polygalacturonase enzyme family (tomato fruit, maize pollen)	61, 62
Cha o 1	38,000	<i>Chamaecyparis obtusa</i>	pectate lyase ³	within Cupressaceae/Taxodiaceae; Amb a 1, Amb a 2; pectate lyase enzyme family	47
Cha o 2	50,490	<i>Chamaecyparis obtusa</i>	polygalacturonase ³	polygalacturonase enzyme family (tomato fruit, maize pollen)	48
Jun a 1	40,000	<i>Juniperus ashei</i>	pectate lyase ³	within Cupressaceae/Taxodiaceae; Amb a 1, Amb a 2; pectate lyase enzyme family	58
Jun a 2	49,354	<i>Juniperus ashei</i>	polygalacturonase ³	polygalacturonase enzyme family (tomato fruit, maize pollen)	52
Jun a 3	21,174	<i>Juniperus ashei</i>	pathogen-related protein (PR-5) ³	wide, among related and distant plant species	51
Jun o 4 ¹	18,000	<i>Juniperus oxycedrus</i>	calcium-binding protein	wide (panallergen?)	63
Jun v 1	39,768	<i>Juniperus virginiana</i>	pectate lyase ³	within Cupressaceae/Taxodiaceae; Amb a 1, Amb a 2; pectate lyase enzyme family	59
Cup s 1 ²	39,835	<i>Cupressus sempervirens</i>	pectate lyase ³	within Cupressaceae/Taxodiaceae; Amb a 1, Amb a 2; pectate lyase enzyme family	–
Cup a 1	37,500	<i>Cupressus arizonica</i>	pectate lyase ³	within Cupressaceae/Taxodiaceae; Amb a 1, Amb a 2; pectate lyase enzyme family	60

¹ Formerly named Jun o 2.

² Sequence submitted in the databases, but not published.

³ Putative, inferred by homology analysis.

Similar features were found in the homologous allergen from *J. ashei*, Jun a 1, cloned by Midoro-Horiuti et al. [58]. Jun a 1, a 346-residue mature protein with a 21-residue signal peptide, was found to have a high degree of sequence identity to Cha o 1 (85%) and Cry j 1 (78.9%) (table 3) and a significant level of identity to Amb a 1 (48.8%) and Amb a 2 (48.3%). Two potential N-glycosylation sites were present, distinct from those found in Cry j 1. The 40,000-dalton recombinant protein was expressed in *Escherichia coli*, and found positive in Western blotting with IgE from *J. ashei*-allergic subjects and anti-Cry j 1 polyclonal antibody. The cDNA for the corresponding allergen in *Juniperus virginiana* pollen, Jun v 1, has been very recently cloned and sequenced, and shows a high degree of homology with the Jun a 1 (96%), Cha o 1 (86%) and Cry j 1 (80%) counterparts [59].

Table 3. Percentage homology among amino acid sequences of the recombinant major allergens (group 1 and 2) from Cupressaceae and Taxodiaceae species

Group 1	Cry j 1	Cha o 1	Jun a 1	Jun v 1	Cup a 1
Cry j 1	–	80	79	80	75
Cha o 1		–	85	86	81
Jun a 1			–	96	91
Jun v 1				–	n.d.
Cup a 1					–
Group 2	Cry j 2	Cha o 2	Jun a 2		
Cry j 2	–	74	71		
Cha o 2		–	82		
Jun a 2			–		

n.d. = Not determined.

Cloned Cup a 1 from *Cupressus arizonica* pollen has been both expressed in *E. coli* and translated in rabbit reticulocytes, in order to enable protein glycosylation [60]. The 346-residue mature protein, with a calculated molecular weight of 37,500 D and three potential N-glycosylation sites, displayed the highest sequence identity to Jun a 1 (91%), followed by Cha o 1 (81%) and Cry j 1 (75%) (table 3), and significant identity ranging from 46 to 51% to the family of pectate lyase-like proteins. The analysis in two different systems, only one of which enabled protein glycosylation, allowed the authors to distinguish between the reactivity of sera from allergic patients. In fact, all seven sera tested in that study recognised recombinant Cup a 1 translated in rabbit reticulocytes, whereas only five of the same seven sera were able to react with Cup a 1 expressed in *E. coli* [60].

As regards group 2 allergens, Cry j 2 was first cloned by applying the same strategy used for Cry j 1. From the analysis of five Cry j 2 cDNA clones [61], a complete sequence of 388 amino acids was deduced, containing one glycosylation site in the C-terminal end. The mature protein had an estimated molecular weight of 42,000 D and showed no homology with Cry j 1. However, database searching revealed that the Cry j 2 amino acid sequence shared significant homology with polygalacturonase enzymes associated with fruit ripening in tomato (40% identity) and also found in maize pollen (34% identity). These data were confirmed by the results of Komiyama et al. [62], who cloned Cry j 2 and evaluated the IgE recognition of the molecule expressed in *E. coli*.

On the basis of the high degree of sequence homology between purified Cry j 2, Cha o 2 and Jun a 2 (table 3), confirmed by rabbit antisera raised against the three allergens, Cha o 2 [48] and Jun a 2 [52] were cloned by using Cry j 2 cDNA fragment as a probe. Cha o 2 and Jun a 2 mature proteins were found to consist of 464 and 453 amino acids, respectively, corresponding to calculated molecular weights of 50,490 and 49,354 D. Jun a 2 showed 71% identity with Cry j 2 and 82% identity with Cha o 2, whereas Cha o 2 had 74% identity to Cry j 2. Two potential N-glycosylation sites were found in the Cha o 2 sequence, and three sites in Jun a 2, but these positions did not correspond with those found in Cry j 2. Since the first of the three motifs was conserved between Cha o 2 and Jun a 2, it is most likely to be a candidate for an actual N-glycosylation site. The conservation of some residues, reported to be a part of the active domain of polygalacturonase, between Cha o 2, Jun a 2 and Cry j 2, strongly suggests that Cha o 2 and Jun a 2 may also belong to this family of enzymes, as already proposed for Cry j 2.

Two other allergens from the *Juniperus* genus have been cloned, and are completely different from group 1 and 2 major allergens.

Recombinant Jun a 3 was cloned from *J. ashei* pollen by PCR with primers deduced from the N-terminal sequence of purified native Jun a 3 [51]. The mature protein, consisting of 199 amino acids with a calculated molecular weight of 21,174 D and one potential N-glycosylation site, was expressed in *E. coli* and found to be recognised by IgE from cedar-hypersensitive patients. The identity analysis performed on the complete sequence confirmed the homology of this pollen allergen with the thaumatin-like PR-5 plant proteins, an ubiquitous family of proteins showing wide similarities among both related and distant species. Very recently, the same authors published a report of the cloning and sequencing of a cDNA for a fragment of the corresponding protein from *J. virginiana* pollen, Jun v 3 [59].

The calcium-binding allergen Jun o 4, formerly named Jun o 2, has been cloned by direct screening with patients' IgE of a cDNA library constructed from the pollen of *Juniperus oxycedrus* [63]. The protein of 165 amino acids, with an estimated molecular weight of 18,000 D, contained four typical Ca²⁺-binding motifs. Jun a 4 showed significant homology with proteins belonging to the calmodulin family (52–55%), restricted to the common Ca²⁺-binding sites. Calcium-binding proteins from different pollens have been described as targets for IgE recognition [64], and this finding could be related to the important role played by Ca²⁺ metabolism in pollen germination and pollen tube growth. For this class of proteins, represented in taxonomically distant species, the designation 'panallergen' has been proposed, since the IgE binding to highly conserved Ca²⁺-binding motifs in these allergens could account for the wide IgE cross-reactivity found beyond the taxonomic family of their plant sources.

The features of both these allergens should be studied to better understand the clinical situation of multiple polysensitised allergic patients and to evaluate the possibility of allergic sensitisation and symptom induction by allergenic sources previously not encountered.

Allergenic Cross-Reactivity

The presence of allergenic cross-reactivity within the Cupressaceae family and with species belonging to the closely related families Taxodiaceae and Podocarpaceae has been widely described since the earliest studies on cypress pollinosis [1, 21, 23, 24, 31, 65]. Data obtained

with whole extracts have been confirmed and implemented at the single allergen level, due to the increasing availability of purified and recombinant allergenic molecules. On the basis of the high IgE cross-reactivity within these families, it is not inconceivable to select a representative extract from one species to be carefully standardised and used for diagnostic and therapeutic purposes [66]. Moreover, the possibility that clinical manifestations may be caused by exposure to cross-reactive species different from those inducing the initial sensitisation must be taken into consideration in times of an increasingly mobile population. Another consequence of the wide cross-reactivity between species and genera is the extension of the pollen season in areas where several Cupressaceae species with close but distinct pollination periods coexist [8].

From a more general point of view, Cupressaceae/Taxodiaceae allergens constitute a particularly useful model to study IgE cross-reactivity, as they have been shown to display different levels of homology.

First of all, the analysis of the complete amino acid sequence of recombinant allergens has proved the close relationship between the 'group 1' and 'group 2' allergenic proteins respectively represented in the species belonging to these families. Common allergenicity and antigenicity between species has been demonstrated also at the level of B and T cell epitopes by studying IgE and mAb reactivity [43] and immunisation in animal models [67, 68].

Secondly, sequence homologies have been found among cypress allergens and proteins from taxonomically unrelated sources. This is the case for the presence of the pectate lyase-like enzymatic site on 'group 1' allergens, shared with the other allergens Amb a 1 and Amb a 2 and with pectate lyase enzymes from various origins. Interestingly, Nakamura et al. [69] recently demonstrated that

Chao 1-specific CD4⁺ memory T cells can survive via stimulation by cross-reactive pectate lyase peptides from unrelated plant sources. Likewise, Jun a 3 shows homologies with the members of the family of thaumatin-like PR-5 plant proteins, an ubiquitous family of proteins, and Jun o 4 may be classified as a calmodulin-like protein. These findings at the molecular level give new support to the earlier observations on the cross-reactivity of Cupressaceae allergenic extracts with taxonomically distinct pollens [31, 70].

Besides the presence of shared protein structures, cross-reactivity among Cupressaceae allergens has also been associated with the glycans represented on the allergenic glycoproteins. These structures, able to bind IgE from allergic patients, have been identified in various instances and have been named cross-reacting carbohydrate determinants (CCDs). CCDs are widely represented not only in allergens from a variety of plants and food of plant origin [reviewed in ref. 71, 72], but also from moulds [73], insects [74] and mammals [75]. These structures could also explain the presence of specific IgE antibodies to allergens in the sera of patients without evidence of previous exposure to the allergenic source. The presence of CCDs recognised by human IgE has been reported for the purified major allergen of *C. japonica*, Cry j 1 [43, 44], and for *C. arizonica* pollen extract [76] and the purified major allergen Cup a 1 [53]. In these cases, deglycosylation treatment of the glycoallergens results in a reduced IgE-binding capability. However, the actual capacity of CCDs to sensitise and to elicit symptoms is still debated and controversial data have been produced. Analysis of the reactivity of glycosylated allergen in a basophil histamine release assay and, whenever possible, in direct skin testing is needed to conclusively address this issue.

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