

Original article

Cloning and expression of a major allergen from *Cupressus arizonica* pollen, Cup a 3, a PR-5 protein expressed under polluted environment

Background: This paper describes the cloning and expression of the *Cupressus arizonica* pollen protein Cup a 3. In addition, we present its modulation under polluted environmental conditions. Species of the Cupressaceae family are important because of their high sensitization prevalence.

Methods: Cup a 3 cloning is based on the sequence of the homologous protein Jun a 3. Cup a 3 was expressed with good yield in the methylotropic yeast *Pichia pastoris*.

Results: Recombinant Cup a 3 (rCup a 3) contains 199 amino acids, 10 potential phosphorylation sites and no glycosylation sites. By immunoblot 63% of cypress allergic patients had specific immunoglobulin E antibodies against rCup a 3 ($n = 104$). This major allergen is homologous to members of the pathogenesis-related proteins (PR-5 group) and contributes to the overall allergenicity of *C. arizonica* pollen. Our results show that the increased expression of Cup a 3 is dependent on the pollution in the area where the pollen has been collected, being higher under polluted conditions.

Conclusions: Cup a 3 is a PR-5 protein derived from *C. arizonica* pollen. The expression of the protein under polluted conditions has a direct incidence on the pollen allergenicity, as has been demonstrated by skin tests and Radioallergo-sorbent test inhibition.

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Key words: Cup a 3; Cupressaceae pollen; pollution and allergenicity; PR-5 proteins.

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Accepted for publication 11 August 2003

Species of the Cupressaceae family (*Cupressus*, *Juniperus*, *Chamaecyparis*, *Callitris*, *Thuja* and *Libocedrus*) are a very important cause of allergies (specially oculo-rhinitis) in various geographical areas including North America, Japan and Mediterranean countries, such as France, Italy and Israel (1). In the case of Spain, Cupressaceae pollen is the predominant pollen in the areas such as Toledo, Barcelona and Madrid from January to April (11%) and it is the main cause of the so called 'winter allergic cold', being 20% the prevalence of positive skin prick test to *C. arizonica* extract (2–4). There is a tendency towards an increase in the reactivity to Cupressaceae pollens because the allergenic load is becoming more intense given that they are frequently used for ornamental purposes and as natural barriers. In addition, the potential adjuvant effect of air pollution may have contributed to this increase. Nevertheless, it has been described several prevalence rates among cypress sensitization with divergent results, all of them collected in geographical areas where cypresses have a high incidence. Whereas some authors think that cypress allergy is underestimated (1, 5, 6), others doing also good epidemiological surveys, stated that the

prevalence of sensitization is low, besides the high allergenic pollen load in the studied areas (7, 8). A possible underestimation of the real prevalence of cypress allergy may be due to the difficulties for allergens extraction from the grains which leads to the lack of good standardization of the extracts. Other possible factors that should be taken in account are genetic protective factors, specially in those populations in close contact with cypresses during centuries (9).

Cupressaceae pollen has two important characteristics: a low protein concentration and its high carbohydrate content. Characterization of the allergenic extract of *C. arizonica* pollen shows that it contains a predominant glycoprotein Cup a 1 (43 kDa), highly homologous with Jun a 1, Cha o 1 and Cry J 1. Cup a 1 was defined as a major antigen with a 72% immunoglobulin (Ig)E recognition and with an active pectate lyase site (10–14).

In this study, we describe the cloning and characterization of another pollen protein Cup a 3. The amino acid sequence of Cup a 3 was found to have a high degree of homology to Jun a 3, a recently described allergen from *Juniperus ashei* (15). Both Cup a 3 and Jun a 3 belongs to

the group of pathogenesis related proteins (PR-5 group). Other important allergens have been described in this group such as thaumatin like proteins from cherry (*Prunus avium*) Pru a 2 and Mal d 2 from apple (*Malus domestica*) (16, 17) with antifungal properties. Also this group of proteins have been found in tobacco leaves infected with virus. They are expressed under stress conditions and it should be taken in to account to be responsible for certain cross reactivities observed in sensitized allergic patients.

Material and methods

Patient recruitment

Allergic patients were from the Allergy Department of Clínica Puerta de Hierro (Dr Domínguez Lázaro and Minguez) in Madrid, ($n = 104$), and Hospital Virgen del Valle (Toledo, Spain) ($n = 75$).

They were diagnosed on the basis of their clinical history, a positive skin test to commercial *C. arizonica* pollen extract (ALK-Abelló, S.A. Madrid, Spain) and the presence in serum of specific IgE antibodies (UNICAP System Test; Pharmacia, Uppsala, Sweden). The average age was 30.7 ± 9 years. A predominance of the female gender was found: 59% vs 41% males. The mean level of total serum IgE was 168.9 ± 8 KU/l and no peripheral blood eosinophilia was observed.

Patients were free from allergen-specific immunotherapy and their previous informed consent to participate in the study was obtained.

Preparation of *C. arizonica* Pollen Extract

Cupressus arizonica pollen was purchased from Allergon (Angelholm, Sweden) or harvested in Toledo (Spain), from trees next to a highway with heavy traffic or in a garden with a low level of pollution, far away from traffic areas and industries. The extracts were obtained as previously described (12). Protein concentration was measured according to the method of Bradford (18).

Isolation of total RNA and cDNA synthesis. Expression of recombinant Cup a 3 in *Pichia pastoris*

Total RNA from *C. arizonica* pollen was used for synthesis and amplification of the Cup a 3 cDNA. Primers corresponding to 5' and 3' ends of the sequence encoding the Cup a 3 protein were designed based on the nucleotide sequence of a homologous protein: Jun a 3. Primers were 5' GGG GGG GCA GAA TAC AAT ACT GTA3' (antisense); 5' GGG GTG AAG TTT GAT ATA AAG AAC3' (sense), (15).

Recombinant Cup a 3 (rCup a 3) was expressed in *P. pastoris* using the pCIZαA vector (Invitrogen, Groningen, The Netherlands). The yeast GS115 strain was electroporated and transformants were selected on yeast extract peptone dextrose agar. A single positive colony was inoculated in a glycerol complex medium and finally cells were resuspended in a buffered methanol complex medium. Expression of rCup a 3 was induced in the presence of methanol. The recombinant protein was purified using immobilized nickel quelate according to the manufacture's instructions (Invitrogen).

The DNA sequence analyses were performed with an AbiPrism sequencer (Applied Biosystems, Foster City, CA). The sequence was compared with the GenBank database for homology to known sequences (Madison, WI).

Animal immunization

Rabbits were injected i.p. with 100 µg of *C. arizonica* crude extract (Allergon) or with 100 µg of rCup a 3 obtained from transformed *P. pastoris* yeast, included in CFA, once a week during 4 weeks.

Immunological characterization

The IgE immunoblot assays were performed as described in the case of Cup a 1 with minor modifications (12).

Rabbit antisera (1 : 5000) produced as described above and peroxidase-labeled goat anti-rabbit IgG (1 : 1000; Caltag, Burlingame, CA) were used in immunoblot assays of the different pollen extracts.

Radioallergosorbent test inhibition

The Radioallergosorbent test (RAST) inhibition was performed using sensitized discs with *C. arizonica* pollen extract (Allergon) (19). The discs were incubated with 50 µl (1 : 3 dilution) of patient serum pool ($n = 30$, CAP class 3 or higher) plus 50 µl of inhibitor extract (prepared with pollens from trees located around the highway or from the garden in a residential area or pollen obtained from Allergon) at different protein concentrations, and later with $\approx 100\,000$ cpm of ^{125} I-anti IgE HE-2 (20). The inhibition (%) of IgE-binding was determined as:

$$\% \text{inhibition} = 100 \times \{1 - [(cpm - cpm100\%) / (cpm0\% - cpm100\%)]\}$$

Where cpm is the disc radioactivity obtained with a determined protein concentration of inhibitor extract, cpm 0% is the disc radioactivity without inhibitor extract, and cpm 100% is the disc radioactivity without serum pool added.

Results

Amplification and sequence analysis of Cup a 3

Polymerase chain reaction (PCR) amplification using primers described above revealed a 597-bp cDNA clone. The deduced amino acid sequence corresponds to a protein of 199 amino acids, with an estimated molecular weight of 21 kDa and a calculated pI of 4.82. Moreover, it presents 10 potential phosphorylation sites. The nucleotide sequence (Fig. 1A) was submitted to the GenBank/EMBL Database (AJ294411, GI-9929163).

The amino acid sequence of Cup a 3 was compared with Jun a 3 (GI-6940771) and a high degree of homology was found (95%). Homology was also found with some members of the PR-5 family: 94% with Jun v 3-1 (GI-8843922), 45% with Pru a 2 (U32440) and Mal d 2 (GI-30316292) and 48% with PR 5d (GI-2981950) Fig. 1B.

Expression of rCup a 3

After cloning of cDNA encoding Cup a 3 allergen in the pCIZαA expression vector, pCIZαA-Cup a 3 was used for transformation of *P. pastoris* GS115 cells, obtaining the efficient extra cellular secretion of rCup a 3. By PCR,

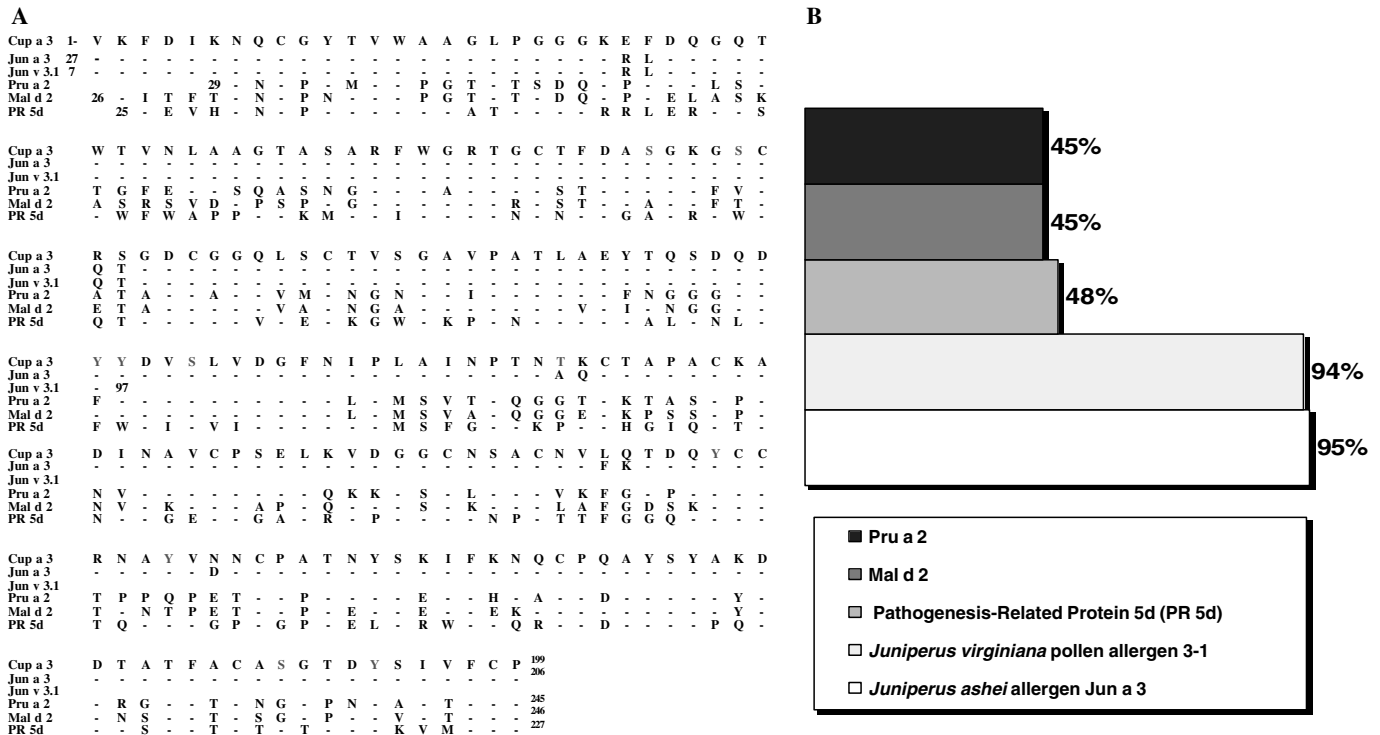


Figure 1. (A) Complete sequence of rCup a 3 (Gene Bank accession number: AJ 294411). Homologies between amino acidic sequences of recombinant Cup a 3 (rCup a 3) and related pollen allergens included in PR-5 like proteins are shown (amino acids between lines are different among sequences studied). (B) Percent homologies between rCup a 3 and some allergenic members of pathogenesis related proteins PR-5.

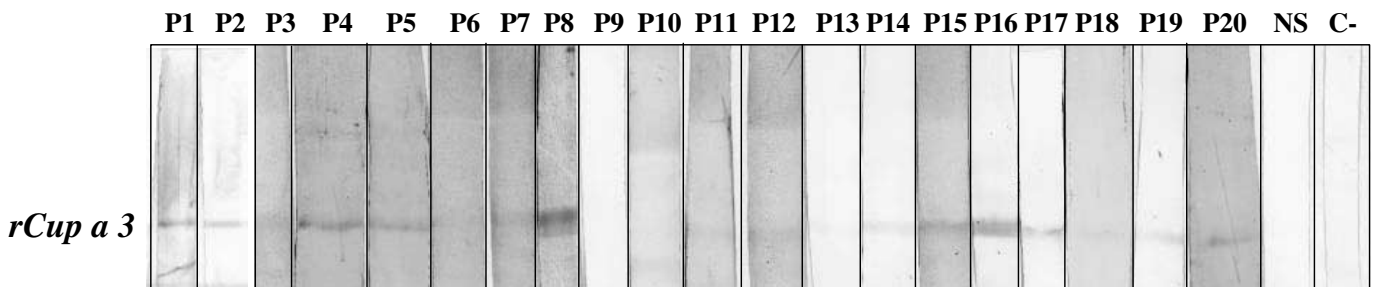


Figure 2. A representative example of immunoglobulin E reactivity by immunoblot using sera from *Cupressus Arizona* sensitized patients and recombinant Cup a 3 as antigen. NS: control using sera from nonrelated allergic patient and C- is the use of buffer instead of sera.

five of 15 clones selected had Cup a 3 cDNA incorporated into the yeast genome and four of these transformants secreted rCup a 3 into the medium. Analysis of the time course of allergen expression in culture supernatants showed that a prominent 23-kDa band was secreted into the media within 24–48 h and rCup a 3 protein was purified using an affinity column.

In order to confirm that the IgE recognition of rCup a 3, the 23-kDa band from one clone was analysed by immunoblot using 104 sera from *C. arizonica* allergic patients. The percentage of sera that recognized the

23 kDa band produced in *P. pastoris* was 63%. Figure 2 shows a representative example.

Effect of environmental conditions on the allergenic potency of *C. arizonica* pollen

Cup a 3 is a member of the PR-5 family, so its expression could be altered by stress conditions. One of the factor that affect the allergenicity is the level of pollution. In order to study this phenomenon we used pollen from areas with a very different pollution conditions: highway

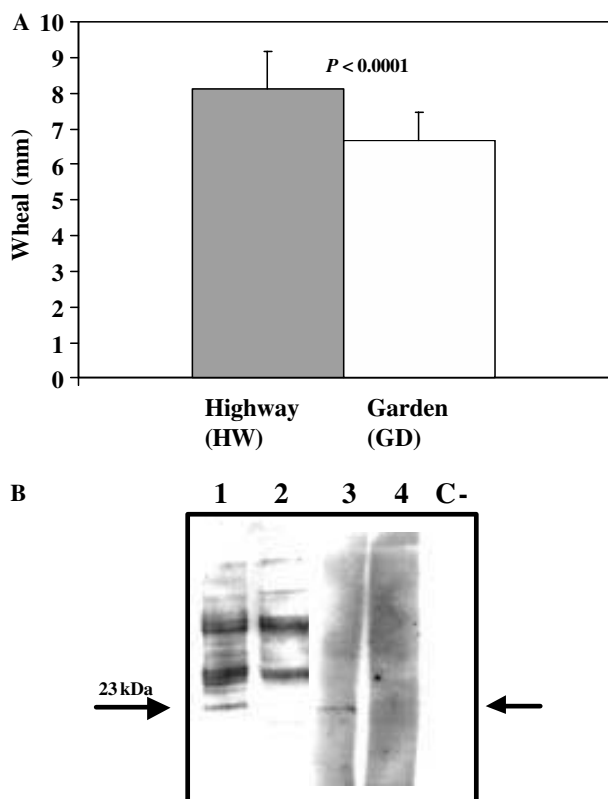


Figure 3. (A) Comparison of reactivity by skin prick test (mm) performed in 75 patients allergic to *Cupressus arizonica* with pollen obtained from trees around a polluted highway in an industrial district (HW) and from nonpolluted garden in a residential area (GD). (B) Immunoblot using different *C. arizonica* pollen extracts obtained from several areas: Lanes 1 and 3 pollen extract obtained in polluted highway (HW) and lanes 2 and 4 in garden far from polluted environment (GD). Blot was developed by incubation with rabbit serum anti-*C. arizonica* whole extract in lanes 1 and 2 or with anti-recombinant Cup a 3 (rCup a 3) antibodies in the case of lanes 3 and 4 (see methods). Arrow points to the 23 kDa rCup a 3 protein. C- is the negative control using buffer.

with heavy traffic around an industrial area (HW) and cypresses planted in garden from a residential neighbourhood far from polluted areas (GD). The results showed that skin prick tests (mm of wheal diameter) obtained from seventy-five patients using HW and GD pollen extracts were statistically different. Mean values for skin tests with extract obtained from a polluted area (HW) was 8.1 ± 1 mm, whereas the wheal area diameter with nonpolluted extract (GD) was 6.6 ± 0.7 mm, $P < 0.0001$ (Fig. 3A).

Figure 3B shows an immunodetection using antigens pollen extracts from polluted (lanes 1 and 3), and from a residential area (lanes 2 and 4). Protein bands were developed with rabbit antibodies obtained by immunization with *C. arizonica* crude extract from Allergon in lanes 1 and 2 or with rabbit antisera obtained by

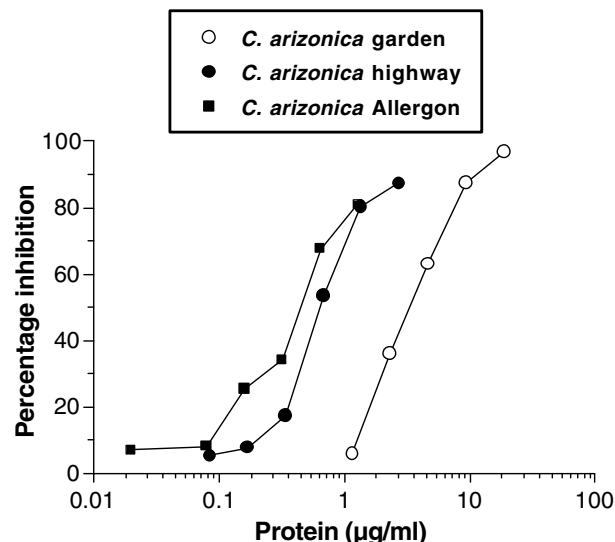


Figure 4. Radioallergosorbent test inhibition using discs sensitised with *Cupressus arizonica* crude extract and a pool of thirty sera from cypress allergic patients with immunoglobulin E specific levels equal or higher than class 3 by CAP system. As inhibitors *C. arizonica* pollen extracts in different concentrations and with different origins were used: garden in residential area, open circles, polluted highway (closed circles) and crude extract from Allergon pollen (squares).

immunization with rCup a 3 (lanes 3 and 4). The 23 kDa band corresponding to Cup a 3 (arrow) only appeared in the extract from pollen obtained under a polluted environment (lanes 1 and 3) and not in the extracts obtained in the clean areas.

Furthermore, a RAST inhibition was performed using sensitized discs with *C. arizonica* pollen extract. Discs were incubated with patient serum pool using as inhibitory extracts different concentrations of extracts obtained in polluted highway, residential garden and *C. arizonica* pollen extract from Allergon. Figure 4 shows that, in accordance with skin test results, the pollen extract from polluted area and the Allergon extract have an allergenic activity of more than five times compared with that obtained with pollen extract from nonpolluted area probably because of the presence of Cup a 3 in the more active extracts.

Discussion

The Coniferales order contains six families, four of which include species that are of interest to the allergist. The *Cupressaceae* family has two closely related genera: *Cupressus* and *Juniperus*. In a previous work we cloned and sequenced a major antigen of *C. arizonica* pollen: Cup a 1 (12). This protein is highly homologous with Jun a 1 the major allergen of *J. ashei* (mountain cedar).

Based on the homology and clinical cross reactivity among these two pollens, we thought that an allergen similar to Jun a 3 (15) could be found in *C. arizonica* pollen. For this reason, we used primers based on the Jun a 3 sequence to clone the *Cupressus* counterpart. The mature protein Cup a 3 consists of 597 nucleotides (199 amino acids) with a calculated molecular weight of 21 kDa. The homology between these two sequences is very high (95%). The identity analysis performed on the complete sequence confirmed the homology of this pollen allergen (Cup a 3) with the thaumatin-like PR-5 plant proteins (Fig. 1), a ubiquitous family of proteins showing wide similarities among both closed and distant species (21, 22). The cDNA sequence of Cup a 3 has 10 potential phosphorylation sites (four Serines, one Threonine and five Tyrosines).

We used *P. pastoris* for protein expression because of the fact that a considerable increase in yield has been previously described for recombinant allergens such as *Cyn d 1* and *Alt a 1* in yeast (23–25). One of the main advantages in producing heterologous proteins as secreted products in *P. pastoris* is the easy isolation of the recombinant molecule from the medium in which it is produced, because it is completely soluble and has a high degree of initial purity due to the very low level of endogenous proteins secreted. Another advantage of this system is the attainment of a native conformation. Allergen expression in *P. pastoris* avoids several problems associated with bacterial expression systems. In our case, using the *P. pastoris* system we have obtained large quantities of biologically active allergen secreted into the medium (1.5–2 mg/ml) that requires minimal purification.

The sera from 104 patients allergic to *C. arizonica* pollen were examined for binding of IgE to Cup a 3 by immunoblot. The percentage of positive sera with IgE antibodies for rCup a 3 was 63%, defining Cup a 3 as the second major allergen in *C. arizonica*. Other important allergen such as Pru a 2 from cherry belongs to pathogenesis-related protein group 5 and has been cloned and expressed in *Escherichia coli*, yielded a product unable to bind IgE in immunoblot (16). One possible explanation given by the authors is that the 16 cysteine residues in Pru a 2 may give a recombinant product incorrectly folded because of the elevated number of disulfide bridges which make the IgE binding highly dependent on the three-dimensional structure. Cup a 3 also have 16 cysteine residues as does the members of PR-5 family and the expression in *P. pastoris* does not alter the IgE binding. The reactivity dependence of the recombinant proteins according to the expression vector is corroborated by a recent paper in which Mal d 2 from the same family PR-5, with eight disulfide bridges, has been expressed in *Nicotiana benthamiana* plants by the use of a tobacco mosaic viral vector. The expressed protein r Mal d 2, conserved not only the IgE reactivity but also its anti fungal activity (17).

Since the 1980s, many clinical, epidemiological and experimental studies have demonstrated that there is a complex interaction between environmental pollution and allergic inflammatory reactions. Type II pollution, characterized by elevation of nitrogen oxides (NO_x), ozone (O₃), tobacco smoke, fine and ultrafine particulate matter, and diesel exhaust particles seems to enhance allergic disease (26–30).

The PR plant proteins represent an important group of human allergens and their expression is up-regulated in plants in response to stressors such as drought, freezing temperature, infection by fungi, viruses or bacteria, ozone and UVB and other stress stimuli. The PR proteins display multiple effects within the plant and can thus be regarded as a part of the plant's defence system (22). Some authors speculate that changes in environmental conditions may affect the expression of PR proteins, thereby contributing to alter the allergenic potency of the pollen (15, 31–33). In the case of Cup a 3, environmental alterations, such as pollution stress, are important. Our results (Figs 3 and 4) show that this allergen is expressed in pollen suffering stress conditions (air pollution because of heavy traffic). By contrast, in pollen obtained from a garden with a low pollution level this protein is not expressed. These results point out the importance of an adequate representations of all allergenic components in the extracts to be used for diagnostic purposes. It seems likely that the extract obtained with commercial pollen that we have used contained Cup a 3, as is shown when we injected it to a rabbit and anti Cup a 3 antibodies, between others, appeared after immunization.

Cupressaceae pollens are one of the most common causes of seasonal allergic disease and the role of *Cupressus* species in winter pollinosis has been clearly demonstrated. The cloning of proteins from this pollen is an important step to discover the characteristics of these proteins and to use them as diagnostic and immunotherapy tools in the future. Our results could account for the increase in the allergenicity of pollen from industrialized areas and therefore try to understand the high prevalence of *C. arizonica* pollen allergy in the last decade.

Acknowledgments

This work has been funded by Fondo de Investigaciones Sanitarias (PI 020517). Also, we appreciate the support given by Fundación Conchita Rábago to Esther Civantos. Esther Lopez is recipient of a fellowship from Becas de Formación en Investigación (BEFI). We are grateful to Dr Ascensión Mínguez (Allergy Unit, Clínica Puerta de Hierro, Madrid) for providing sera and data from allergic patients. Dr Pilar Palomino, Blanca Cárdbaba and Soledad Gallardo (Fundación Jiménez Díaz, Madrid) are also acknowledged for helpful criticisms and document revision. We also thank Ms Linda Puebla for language revision.

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