Identification of a New Allergen from Amaranthus retroflexus Pollen, Ama r 2

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ABSTRACT
Background: Pollinosis from Amaranthus retroflexus pollen is a common cause of respiratory allergy in Iran with a high positive rate (68.8%) among Iranian allergic patients. The aim of the present study was to evaluate the allergenicity of the A. retroflexus pollen profilin.

Methods: Using sera from twelve patients allergic to A. retroflexus pollen, IgE-binding proteins from the A. retroflexus pollen extract was identified by immunoblotting. The cDNA of A. retroflexus pollen profilin was amplified, then cloned into the pET-21b (+) vector, expressed in Escherichia coli, and finally purified by metal affinity chromatography. The IgE-binding capacity of the recombinant protein was then analyzed by the ELISA, immunoblotting, and inhibition assays, as well as by the skin prick test (SPT).

Results: Immunoblotting results indicated a 14.6 kDa protein with IgE-reactivity to 33% (4/12) among A. retroflexus pollen-allergic patients. Nucleotide sequencing of the cDNA revealed an open reading frame of 399 bp encoding for 133 amino acid residues which was belonged to the profilin family and designated as Ama r 2. A recombinant Ama r 2 (rAma r 2) was then produced in E. coli as a soluble protein which showed a strong IgE-reactivity via ELISA confirmed by the SPT. Inhibition experiments revealed high IgE cross-reactivities with the profilins from other plants.

Conclusions: The profilin from the A. retroflexus pollen, Ama r 2, was firstly identified as an allergen. Moreover, rAma r 2 was produced in E. coli as a soluble immunoreactive protein with an IgE-reactivity similar to that of its natural counterpart.

KEY WORDS
allergen characterization, Ama r 2, Amaranthus retroflexus, cloning, profilin

INTRODUCTION
Immunoglobulin E (IgE)-mediated allergy to pollens from the Amaranthaceae is common in semi-desert countries such as Saudi Arabia, Iran, and Kuwait.1-4 Particularly among Iranian allergic patients, allergy to A. retroflexus (redroot pigweed) pollen is well documented with the high positive rate of 68.8%.4

In 1985, Lombardero et al. reported that pollens from the Amaranthaceae family, including A. retroflexus, Lamb’s Quarter (Chenopodium album), and Russian Thistle (Salsola kali), contained significant allergens with molecular weights (MWs) of 35 and 14 kDa. In addition, it was shown that all of these pollens contained cathodic migrating allergens.5 In 1995, Würtzen et al. introduced eleven allergenic proteins from the A. retroflexus pollen including three proteins with MWs of 14, 15.5 and 17 kDa.6 Recently, we showed an allergenic protein from the A. retroflexus pollen with an apparent MW of 14-15 kDa which had not yet been identified.7

Profilin is a well-known ubiquitous cytoskeleton protein in eukaryotic cells. It is presumably a link between the microfilament system and signal transduction pathways.8 Profilin was first recognized as an allergen in birch pollen and named Bet v 2.9 Up to now, profilins have been recognized as the allergens in several pollens, as well as some plant-derived foods.10

In this study, a new allergen of A. retroflexus pollen was identified which was designated as Ama r 2.
METHODS
PATIENTS
Twelve adult respiratory allergic patients were enrolled from the Outpatient Allergy Clinic of Mashhad University of Medical Sciences. After getting informed consent, patients were asked to complete a detailed questionnaire. They were regarded to have a history of allergy if they stated at least one eye, nasal, or respiratory symptom to common allergens such as house dust, domestic animals, food, or pollen. Patients were also evaluated by clinical examination and skin-prick test with common aeroallergens. Two control subjects with no history of allergy and negative SPTs were also included in the study. The study was approved by the Human Ethics Committee of the university.

SKIN PRICK TEST
Skin prick test (SPT) was performed according to Dreborg’s Method. Tests were performed with 5 μl of each of A. Retroflexus, C. album, and S. kali pollen extracts (All from Hollister-Stier Laboratories LLC, Spokane, WA, USA) placed on the ventral side of the forearm with standard lancets. After 10 to 15 minutes, the region where the allergen was applied was observed for wheal (raised area) and flare (redness). Histamine as a positive control was also used to make confident of no anti-histamines have been taken which can interfere with the testing results. As a negative control, 50% glycerin solution was applied to make sure the patient was not dermographic and falsely being identified as sensitive to A. retroflexus. A wheal at least 3 mm larger in diameter than the negative control, surrounded by an erythema, was considered as a positive skin test.

TOTAL EXTRACT PREPARATION
Pollen from A. retroflexus was purchased from Allergon AB (Välinge, Sweden) and extracted in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) in 1:20 w/v overnight on a shaker, at 4°C. Then it was centrifuged at 7150 x g for 20 min, and the clear supernatant was dialyzed for 16 h at 4°C against Tris-HCl 1 0.02 M, pH 8.8. The filtrate was then lyophilized and stored at -70°C. Lyophilized samples were reconstituted in distilled water (1/10 w/v) and the protein content of which was determined using the Bradford’s method.

ENZYME- LINKED IMMUNOSORBENT ASSAYS (ELISAs)
Total serum IgE levels were measured by means of a commercially available ELISA kit (Radim, Pomezia Terme, Italy) according to the manufacturer’s instructions. To measure the levels of specific IgE to A. retroflexus pollen in patients’ sera, an indirect ELISA was developed as described earlier. Briefly, 2 μg of A. retroflexus pollen extract in 100 μl carbonate buffer (15 mM Na2CO3 and 35 mM NaHCO3, pH 9.6) per well of a 96-well microtiter plate (Nunc MaxiSorp™, Fisher Scientific, Pittsburg, PA, USA) was incubated overnight at 4°C. Each well was then blocked for 1 h at 37°C with 150 μl of 2% Bovine Serum Albumin (BSA) in PBS following by incubation for 3 h with 100 μl of serum at room temperature with shaking. Each well was then incubated for 2 h at room temperature with 1:1000 dilution of biotinylated goat anti human IgE antibody (Kirkegaard & Perry Laboratories, MD, USA) in 1% BSA. Each incubation step was followed by 5 washes with PBS-T (PBS containing 0.05% Tween 20). One hundred microliters of a 1:30000 dilution of horseradish peroxidase-conjugated streptavidin (Bio-Rad, MD, USA) were then added to each well. Following 5 washes with PBS-T, 100 μl of chromogenic substrate was added to each well and the plate was incubated for 15 min in the dark. Color development was stopped by the addition of 20 μl of 3 M hydrochloric acid. The plate was read at 450 nm with an ELISA reader. Optical density (OD450) greater than three times the median values of negatives control were considered to be positive.

SDS-PAGE, IgE IMMUNOBLOTTING AND INHIBITION ASSAYS
Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the A. retroflexus pollen extract was performed according to Laemmli using a 15% acrylamide separation gel under reducing conditions. Separated protein bands from the electrophoresis of A. retroflexus pollen extract were electrotransferred to polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore Corp., Bedford, MA, US), as described by Assarehzadegan et al. Inhibition experiments were performed using two recombinant allergens which were previously expressed in our lab and belonged to profilin family including the recombinant Cuc m 2 (from Cucumis melo fruit), and the recombinant Che a 2 (from C. album, unpublished data). Ten micrograms of the purified rChe a 2, 3.3 μg of the purified rCuc m 2, or 7 μg of BSA (Negative control) were pre-incubated with a pooled serum (from patient 1, 5 and 6 as in Table 1) for 1 h at 37°C. The pre-adsorbed sera were then used for immunoblotting assays. BSA was also used for inhibition as control.

CLONING AND SEQUENCING
Total RNA was extracted from the pollen basically by the method of Chomczynski and Sacchi. First Strand of cDNA was synthesized using RevertAid™ First Strand cDNA Synthesis Kit (Fermentase, Vilnius, Lithuania) according to the manufacturers’ instructions. PCR was performed using two degenerate primers which hybridized with a DNA whose se-
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**Table 1** Clinical data, specific IgE levels and SPT responses of the selected patients with allergy to *A. retroflexus* pollen

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age (years)/sex</th>
<th>Clinical history</th>
<th>Total IgE IU/ml</th>
<th><em>A. retroflexus</em> pollen extract</th>
<th>Recombinant Ama r 2</th>
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1^AR, Allergic rhinitis; RC, rhinoconjunctivitis.

1^The mean weal diameters are displayed in mm². Histamine diphosphate (10 mg/ml)-positive control; Glycerin-negative control.

1^Determined in ELISA as OD (optical density) at 450 nm.

The coding region from Ama r 2 was amplified with *Pfu* DNA polymerase (Fermentase), using two specific primers. According to the nucleotide sequence of Ama r 2 (GenBank accession number: FJ899746), the sense primer (5'-TCCCGGGGCCGCAATGTCGTCGAGCGTATGT-3') is complementarily overlapped with the first six codons and introduces a *NdeI* restriction site (underlined). The antisense primer (5'-TCCCTCGAGGTAACCTGCTGAGGT-3') mimics the last six codons, excluding the stop translation codon, and introduces an *XhoI* restriction site (underlined). After PCR amplification, the 399-bp product was ligated into pET-21b (+), (Novagen, WI, USA). The resulting plasmid containing the Ama r 2 gene in pET-21b (+) was used to transform the host cell *E. coli* BL21 (DE3) (Novagen). Cells were grown at 37°C until to an OD_600_ of 0.4 was reached, when expression was induced by the addition of 0.2 mM IPTG. After induction, in order to improve the solubility of the recombinant protein, the cultures were incubated at 18°C and the cells were allowed to grow for a period of 12 h. The cells were then disrupted by freezing in liquid nitrogen following by thawing at 37°C. The produced rAma r 2 was purified from the soluble phase of lysate by an Ni-NTA agarose (Invitrogen, CA, USA) according to the manufacturer’s instructions. The protein concentration was determined according to the Bradford’s method. The purified protein was then subjected to reducing SDS-PAGE and immunoblotting.

**rAma r 2-SPECIFIC ELISA**

In order to determine the serum IgE levels to the purified rAma r 2, an indirect ELISA was developed as described above, except that the wells of the ELISA microplate were coated with 100 μl of the purified rAma r 2 at a concentration of 10 μg/well in coating buffer (15 mM Na_2_ CO_3_ and 35 mM NaHCO_3_, pH 9.6) overnight at 4°C. Results were expressed as optical density (OD) units. Based on the mean value of two normal sera OD_450_ greater than three times the median values of negatives control were considered to be positive.

**rAma r 2-SPECIFIC IMMUNOBLOTTING AND INHIBITION ASSAYS**

rAma r 2 was electrophoresed and transferred on a PVDF membrane as described above. Sixty microliters of pooled serum (from patients 1, 5 and 6) was
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Fig. 1 (A). SDS-PAGE of the crude extract of the A. retroflexus pollen on a 15% acrylamide gel. Lane MW, low molecular weight (Amersham, Buckinghamshire, UK); (B). IgE-Immuno blot of A. retroflexus pollen extract using allergic patients’ sera; Lane MW, low molecular weight (Amersham); Lanes 1-12, probed with individual allergic patients’ sera; Lane C, probed with a pooled serum from control subjects.

mixed with 5 μg of the purified rAma r 2, rChe a 2, rSal k 4 (a recombinant profilin from S. kali pollen produced in the same lab), or with BSA. Thereafter, samples were diluted to 600 μl and incubated 2 h at 37°C on a shaker. The pre-adsorbed sera were then used for immunoblotting assays.

SPT WITH rAma r 2
Different concentrations of the purified rAma r 2 were mixed with equal volume of glycerin and used for SPTs using two volunteer allergic individuals. Final concentrations of rAma r 2 used for SPTs were 0.4 and 0.2 μg/ml.

RESULTS
PATIENTS
Twelve patients suffering from respiratory allergy were included in our study. Case histories with respect to respiratory allergy are summarized in Table 1. Rhinitis and rhinoconjunctivitis was the most prominent clinical manifestations among these patients. All patients showed positive SPTs to the A. retroflexus pollen extract (Table 1) as well as the C. album and the S. kali pollen extracts (data not shown). Sera from nine of the twelve (75%) patients showed total IgE concentrations more than 100 IU/ml.

IgE-BINDING PROFILE OF THE A. retroflexus POLLEN WITH HUMAN SERA
The protein profile of A. retroflexus crude extract on a 15% acrylamide gel showed at least nine bands in the MW range of 10-70 kDa; including 85, 66, 50, 45, 39, 25, 18, 15, and 10 kDa (Fig. 1A). Specific IgE binding fractions probed against sera from all twelve allergic patients along with a pooled serum from the two control subjects are shown in Figure 1B. Six IgE binding protein fractions of 85, 45, 39, 18, 15, and 10 kDa were detected from the blot. Among all twelve samples, sera from four patients (patient number 1, 5, 6 and 10; as in Table 1) recognized a band with the apparent MW of 14-15 kDa (Fig. 1) which were estimated by Kodak1D Image (Eastman Kodak) as 14.6 kD. However, when the pooled sera of normal volunteers were used no IgE binding fractions was observed. According to the results of the specific ELISA, 9/12 of patients showed specific IgE levels of A. retroflexus pollen (Table 1).

INHIBITION OF IgE REACTIVITY TO Ama r 2 BY PROFILINS FROM OTHER PLANTS
Immunoblotting-inhibition experiments showed that both rChe a 2 and rCuc m 2 were able to inhibit IgE binding to Ama r 2 in A. retroflexus pollen extract (Fig. 2). According to densitometric analysis, rChe a
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**Fig. 2** IgE immunoblot inhibition with *A. retroflexus* pollen extract. Lane MW, low MW (Amersham); Lane 1, *A. retroflexus* protein strip probed with pooled sera from patients’ number 1, 5, and 6, with reactivity to 14.6 kDa components, without inhibitor; Lane 2, *A. retroflexus* protein strips incubated with pooled sera containing 10 μg of purified rChe 2 as inhibitor; Lane 3, *A. retroflexus* protein strips incubated with pooled sera containing 3.3 μg of purified rCuc m 2 as inhibitor; Lane 4, *A. retroflexus* protein strips incubated with pooled sera containing 280 μg of *A. retroflexus* pollen extract as inhibitor; Lane 5, *A. retroflexus* protein strip probed with 1% BSA, without inhibitor.

2 and rCuc m 2 were able to inhibit 87.1% and 93.2% of the IgE-binding band (14.6 kDa) detected in *A. retroflexus* pollen extract, respectively.

**cDNA AMPLIFICATION AND SEQUENCE ANALYSIS OF Ama r 2**

cDNA amplification of Ama r 2 using degenerate primers resulted in a single 399 bp fragment which was cloned into pTZ57R/T. Two of these clones were sequenced. Sequence analysis revealed that the PCR product correspond to a 399 bp open reading frame (GeneBank accession number FJ899746) which encodes Ama r 2, a 133 amino acid peptide belonging to the family of profilins. It had an average MW of 14.6 kDa and a theoretical isoelectric point value of 4.32. It was also submitted to WHO/IUIS Allergen Nomenclature Sub-Committee.

Amino acid sequence of Ama r 2 was compared with those of profilins from other plants. The selected plant-derived profilins were Ama v 1 (*Amaranthus viridis*), Sal k 4 (*S. kali*), Hev b 8 (*Hevea brasiliensis*), Cuc m 2 (*C. melo*), Che a 2 (*C. album*), and Cro s 2 (*C. sativus*). High levels of sequence identity and similarity (87% and 93%, respectively) were detected between the Ama r 2 and the Sal k 4. The most conserved residues showed involvement in specific biological functions and structural roles (Fig. 3).

**EXPRESSION AND PURIFICATION OF rAma r 2**
The Ama r 2 was expressed in *E. coli* BL21 (DE3) as a fusion protein with a His6-tag to permit affinity purification on a Ni²⁺-charged resin. The target protein containing His6-tag in the C-terminus was present in a soluble form in the supernatant, where it was further purified by Ni²⁺ affinity chromatography to yield purified, homogenous protein.

The purified rAma r 2 was quantified using the Bradford's protein assay which showed that approximately 2 mg of recombinant protein had been purified from one liter of the bacterial expression medium. SDS-PAGE (Fig. 3) showed that the MW of the expressed product was about 18 to 19 kDa, which confirmed that the recombinant protein was expressed as a fusion protein tagged with 6 histidine residues.

**IgE REACTIVITY OF rAma r 2**

Using an indirect ELISA, the specific IgE levels to the purified rAma r 2 were determined with twelve individual patients’ sera (Table 1). All except one patient (No. 12) showed significantly elevated specific IgE levels to *A. retroflexus* pollen extract and to purified rAma r 2 (Table 1).

A pooled serum from three patients allergic to *A. retroflexus* pollen (patients 1, 5 and 6) was tested by immunoblotting assays for IgE reactivity to rAma r 2 and showed a reactive band with the MW of 18 to 19 kDa (Fig. 4, Lane 1).

Inhibition experiments demonstrated that pre-incubation of serum samples with rAma r 2, rChe a 2 or rSal k 4, completely inhibited IgE binding to rAma r 2 (Fig. 4, Lanes 2-4).

The results of SPTs with rAma r 2 on two volunteer patients confirmed the IgE reactivity of rAma r 2. rAma r 2 was used at concentrations of 0.4 and 0.2 mg/ml which elicited wheals with the mean diameters of 6 and 3 mm, respectively (Fig. 5).

**DISCUSSION**

In this study, the characterization, cloning and expression of the first allergen of the *A. retroflexus* pollen is reported. This allergen was shown to be a part of the profilin family and was designated as Ama r 2. Previously, there were several reports of the allergenicity of profilins in pollens of the Amaranthaceae/Chenopodiaceae family. In 2003, Che a 2 was isolated as a significant protein allergen from the *C. album* pollen extract. It was also indicated that this protein displayed structural and functional properties similar to those of profilins. In 2007, Ama v 1 was reported as an allergen of the *A. viridis* pollen. It showed a high similarity to the profilin family (unpublished data). In 2010, an allergenic profilin molecule from the *S. kali* pollen was identified and designed Sal k 4. Concur-
Comparison of the Ama r 2 amino acid sequence with allergenic profilins from other plants. The amino acid sequence identity and the similarity of Ama r 2 (ACP43298) with other members of the profilin family are indicated at the end of each amino acid sequence.

A. viridis (Ama v 1, ABW37744), S. kali (Sal k 4, Q0145223), H. brasiliensis (Hev b 8, CAA75312), C. melo (Cuc m 2, AAW69549), C. album (Che a 2, ACR77509), and C. sativus (Cro s 2, AAW81034). The highly conserved residues in plant-derived profilins involved in biological and structural functions (Poly-L-proline binding, fold conservation, actin binding site and stabilized turn) are shaded in gray.

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Immunoblotting inhibition. Lane MW, low MW (Amersham); Lane 1, rAma r 2 protein strip incubated with pooled serum without inhibitor; Lane 2, rAma r 2 protein strip incubated with pooled serum containing 5 μg of rChe a 2 as inhibitor; Lane 3, rAma r 2 protein strip incubated with pooled serum containing 5 μg of rAma r 2 as inhibitor; Lane 4, rAma r 2 protein strip incubated with pooled serum containing 5 μg of rSal k 4 as inhibitor.

The results of SPTs with rAma r 2 on a volunteer allergic patient. 1) Histamine, 2) Glycerin, 3) A. retroflexus pollen extract (Hollister-Stier Laboratories LLC, Spokane, WA, USA), 4) rAma r 2 (0.4 mg/ml), 5) rAma r 2 (0.2 mg/ml).

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Current with these studies, one of our investigations reported an IgE binding protein with an estimated MW of 15 kDa which was detected by immunoblotting of the three related pollen extracts including A. retroflexus, C. album and S. kali. It was also suggested that this protein band is a common minor allergen of the three tested pollens.7

In this study, the apparent MW of Ama r 2 was 14.6 kDa. Via immunoblotting, this protein was recognized in about one third of patients allergic to the A. retroflexus pollen extract. So far, different MWs for profilins from different plant sources have been reported, such as 15.0 kDa in celery,18 14.4 kDa in date palm pollen,19 and 14.0 kDa in oranges.20 These discrepancies could be explained by diversities in a few amino acid residues or variations in the methods of measuring MWs.

Immunoblot inhibition studies showed cross-reactivities between Ama r 2 and other plant profilins, including Che a 2, Sal k 4, and Cuc m 2 (Fig. 2, 4). In addition, amino acid homology indicated a close rela-
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Allergies demonstrated extensive IgE cross-reactivities among profilins from different plants. In addition, several previous studies demonstrated extensive IgE cross-reactivities among profilins from different plants.

According to the results of immunoblotting inhibition assays, both rChe a 2 and rCuc m 2 inhibited the IgE reactivity of the natural Ama r 2 (Fig. 2). Considering that lower amounts of rCuc m 2 produced a more intensive inhibition than higher amounts of rChe a 2, it was suggested that IgE cross-reactivity between Ama r 2 and Cuc m 2 exceeded that between Ama r 2 and Che a 2. These results are in contrast to the phylogeny of these plants, since both A. retroflexus and C. album are from the Amaranthaceae family while C. melo is a fruit which is related to another division of plants. Furthermore, the amino acid sequence homology of profilins from different plants showed higher identities and similarities between Ama r 2 and Cuc m 2 than those of Ama r 2 and Che a 2 (Fig. 3). Therefore, it appears that, although Ama r 2 and Cuc m 2 belong to unrelated plants, their IgE binding epitopes are very similar. Concomitant with these results, other studies have reported IgE cross-reactivities of food allergens and pollens, the so-called pollen-food allergy syndrome, in which there are common IgE epitopes in pollens and foods. Especi- tally, profilin molecules were indicated to be resistant to gastric digestion and so producers of IgE cross-reactive responses.

Finally, rAma r 2 was successfully expressed in E. coli as a soluble molecule. During the process of rAma r 2 expression and after induction with IPTG, the temperature of the culture medium was lowered to 18°C to obtain a high amount of the soluble form of the protein. This was also showed by our previous studies.

The recombinant Ama r 2 showed IgE reactivity to patients’ sera via ELISA and immunoblotting, which also confirmed the correct folding of the molecule. The results of SPTs with the recombinant protein revealed an in vivo IgE reactivity as well. Taken together, it seems that rAma r 2 preserves conformational IgE binding epitopes similar to those of its natural counterpart in A. retroflexus pollen extract.

During the last decade, purified native or recombinant allergens have shown promise as a safe and reliable diagnosis and therapy of allergic disorders. For this propose, the identification of relevant allergens using appropriate techniques is a crucial step.

In conclusion, the first allergen from the A. retroflexus pollen was identified as belonging to the family of profilins and designated as Ama r 2. This allergen was successfully expressed in E. coli as a soluble recombinant protein (rAma r 2). It was also shown to be IgE cross-reactive with several profilin molecules from other plants. The study of the tertiary structure may be helpful in identifying cross-reactive epitopes.

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