

Cloning and Expression of the Allergen Cro s 2 Profilin from Saffron (*Crocus sativus*)

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ABSTRACT

Background: Profilin is a panallergen that is recognized by IgE in allergic patients. Allergy to saffron (*Crocus sativus*) pollen has been described in people exposed to its pollen. Saffron contains a profilin that may cause allergic reactions in atopic subjects. The aim of this study was to describe the cloning, expression and purification of saffron profilin from pollen.

Methods: Cloning of saffron profilin was performed by polymerase chain reaction using specific primers from saffron pollen RNA. Expression was carried out in *Escherichia coli* BL21 (DE3) using a vector pET-102-TOPO. A recombinant fusion protein was expressed and the recombinant profilin was purified by metal precipitation. Immunological characterization was performed by immunoblotting experiments.

Results: The 34 kDa- recombinant saffron profilin, Cro s 2, as a fusion protein was purified. Immunoblotting tested with the sera of allergic patients showed a specific reaction with the recombinant Cro s 2 band.

Conclusions: The sequence of Cro s 2 showed a high degree of identity and similarity to other plant profilins and the recombinant saffron profilin, Cro s 2, may be used for target-specific diagnosis and structural analyses and investigation of cross reactivity of Cro s 2 with other plant profilins.

KEY WORDS

allergy, cloning, Cro s 2, profilin, recombinant allergen, saffron

INTRODUCTION

Saffron (Zaafaran) is a common food additive. Saffron is now cultivated in Turkey, India, China, Italy and Greece, but Iran and Spain are the largest producers, accounting together for more than 80% of the world's production.¹ The pollen of *Crocus sativus* (saffron plant) as an aeroallergen is known to be an important cause of pollinosis in cultivation areas of saffron.² Symptoms in the nose (sneezing, blockage, and running), eyes (itching, redness, tears, and swelling), mouth and throat (itching and dryness), and chest (breathlessness, cough, wheezing, and tightness) are very common during saffron flowering in atopic people who are exposed to its flowers. Allergy to the saffron flower and its clinical significance were studied, previously.¹⁻³ The influenza-like- symptoms during

saffron flowering in saffron farm areas in Iran has been reported in a study which measured the involvement of saffron pollen as an aeroallergen.⁴ The positive skin prick tests with saffron extract in atopic subjects and the presence of the serum saffron specific-IgE was confirmed with RAST.² Also, elevated levels of IgE antibodies against pollens have been found in the sera of sensitized people.¹ Specific saffron pollen IgE and IgG subclasses were detected significantly higher in the allergic subjects than the control group. We reported, the existence of a positive correlation between specific IgE and skin reaction by prick test in atopic subjects ($R = 0.433$). A negative correlation between specific IgE and IgG4 subclass was also found ($R = -0.576$).¹ All these reports confirmed involvement of an immunologic and IgE-mediated reaction. Saffron may induce symptoms of food allergy in

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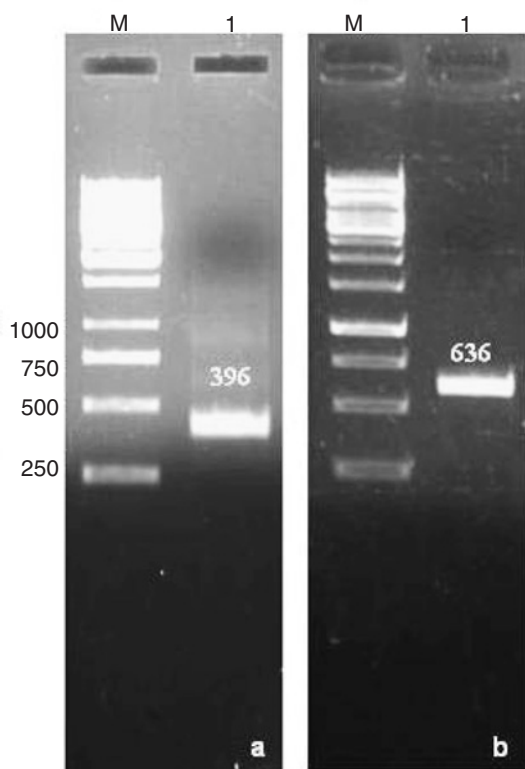


Fig. 1 PCR products were subjected to agarose gel electrophoresis. **a.** Electrophoresis of the PCR-amplification of cDNA encoding Cro s 2 from pollen. M: PCR marker, size in bp (1 Kb, Fermentas), 1: PCR product of 396-bp Cro s 2. **b.** Verification of pET 102/D-TOPO expressing Cro s 2 vector after cloning of the 396- bp PCR product using specific primers of TOPO Kit. M: PCR marker, size in bp (1 Kb, Fermentas), 1: PCR product of 636-bp.

sensitized individuals.⁵ Sensitization to aeroallergens of saffron can be defined as an occupational allergy which is one of the most frequent causes of asthma.^{2,6}

No allergens from this plant have yet been fully characterized. A 15.5 kDa profilin-like allergen was detected from the pollen and stamens, but in very low levels. Several proteins in the 27-67 kDa range were mainly involved in the IgE-binding inhibition study.²

Profilin is a small (12-16 kDa), ubiquitous eukaryotic protein that is recognized as allergenic in pollens, vegetables, and fruits. Profilin can also be allergenic in often as a consequence of cross-reactivity with IgE directed to pollen profiling.^{7,8} Profilin sequence homology is high (70% to 85%) among plants. Many plant profilins exhibit significant cross-reactivity, apparently because of conserved amino acid sequences and shared IgE-reactive epitopes, prompting the designation of profilins as pan-allergens.^{7,9} Previously saffron pollen profilin was purified from the crude extract by size exclusion chromatography.¹⁰ Due to the difficulty of purification of profilin by biochemical

methods, in this study we describe the cloning, expression and purification of the panallergen profilin from saffron pollen and the immunological characterization of the recombinant allergen.

METHODS

HUMAN SERA

Sera from 10 individuals allergic to saffron pollen confirmed by prick test with total extract of saffron were acquired.

A serum pool was obtained by mixing equal-volume aliquots of the individual sera, and then was stored in aliquots at -80°C .

POLLEN EXTRACT

Saffron pollen extracts were prepared following methods previously described.¹ Briefly, pollens were collected from pistils of saffron. Defatting was done using cold acetone (1/10 w/v) and shaking for 16 hours. Acetone was removed by suction and then phosphate-buffered saline (PBS, pH = 7.4, 0.01 M) containing 20 mM EDTA was added to the samples and shaken for 18 hours. After centrifuging at 5600 g for 30 minutes the supernatant of this mixture was dialyzed in PBS for 48 hours. All of extraction steps were performed at 4°C .

ISOLATION OF RNA, PCR-AMPLIFICATION OF cDNA ENCODING CRO S 2 FROM POLLEN

Total RNA was extracted from 1 g of fine powder from pistils and stigma of saffron plant grounded under liquid nitrogen by means of the ConcertTM plant RNA purification kit (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from 2 μg total RNA using a first-strand cDNA synthesis Kit (Fermentas, Ontario, Canada) with an Oligo (dT) 18 as primer. The saffron profilin coding region was amplified with *Pfu* DNA polymerase (Fermentas), using two degenerate primers which were based on the sequence similarity between conserved regions of profilin among plants: primer 1, 5'-ATGTCGTGGCARRC BTAYGT-3' and primer 2, 5'-ATRADCCYTGNTCDAT NAGRTART-3'.¹¹ The PCR product was sequenced (TIB MOL BIOL, Germany) and using two specific primers, according to the obtained sequence of Cro s 2 (5'-CACCTATCTCGAGATAGCCCTGTTTCGATGAGGTA-3', 5'-TGACATATGTCGTGGTGGCAAACGTA CGT-3'; underlined sequence indicates a 4-nucleotide addition) the saffron profilin, Cro s 2 was amplified.

CLONING

The predicted 396-bp PCR product was purified from agarose gel using DNA extraction Kit (MBI Fermentas, Lithuania) and was cloned into the pET 102/D-TOPO expression vector (Invitrogen), which expresses a recombinant fusion protein containing thioredoxin at the N-terminal end and a His-tag at the C-terminal end. The resulting construct was trans-

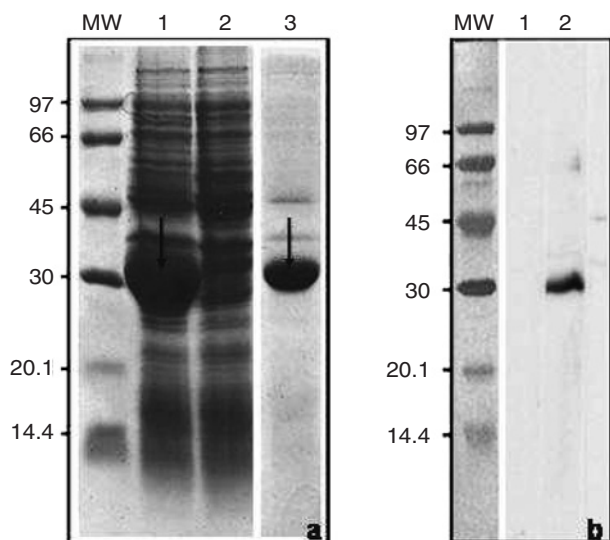


Fig. 2 SDS-PAGE (12%) for analyzing of Cro s 2 expression in bacteria lysate with Commassi blue staining and immunoblotting, Lane MW: molecular weight marker, size in kDa. **a.** Lane 1: An expressed 34-kDa band is seen after IPTG induction. Lane 2: before IPTG induction. Lane 3: A sample of purified recombinant Cro s 2. Arrows show the rCro s 2. **b.** Lane 1: Immunoblotting of purified r Cro s 2 protein with a pooled non allergic serum as negative control, Lane 2: Immunoblotting of purified r Cro s 2 protein with a pooled saffron allergic serum, the 34-kDa band was reactive.

formed into TOP10 *E. coli* cells. Colonies were selected and analyzed on ampicillin agar. The fidelity of the cloned product was verified by PCR reaction using specific primers of TOPO Kit. The purified plasmid was sequenced (TIB MOL BIOL).

RECOMBINANT PROTEIN EXPRESSION AND PURIFICATION

For expression of Cro s 2, the pET-102- TOPO constructs were transformed in *E. coli* (DE-3) competent BL21 Star cells (Invitrogen) and protein synthesis was induced with 1 mM IPTG (isopropyl β -D-thiogalactoside) for 5 hours at 37°C. Bacteria were harvested by centrifugation (3000 g, 20 minutes, 4°C) and stored at -80°C. The pellet from a 1-litre bacterial culture was resuspended in lysis buffer (50 mM NaH₂PO₄, 500 mM NaCl and 2 mM imidazole, pH 8) and subjected to three freeze-thaw cycles using liquid nitrogen (frozen 3 times in liquid nitrogen). Recombinant Cro s 2 (rCro s 2) was purified with a metal precipitation method from the insoluble phase.¹²

IMMUNODETECTION

Purified rCro s 2 and total extract of saffron pollen extract were then subjected to reducing 12% (w/v) SDS-PAGE according to Laemmli,¹³ and electroblotted on to polyvinylidene difluoride (PVDF) membranes

(Immobilon-P, Millipore Corp., Bedford, MA, USA). The SDS-PAGE profile of the proteins subsequently was used for immunoblots.

After blocking with 2% BSA for 16 hours at 4°C, the blots were incubated for 16 hours at 4°C with the 10 sera of allergic patients individually and then pooled, diluted 1 : 4 in PBS, for 3 hours at room temperature. The blots were then incubated with anti-human IgE biotin-conjugated goat antibody (Sigma, USA) (1 : 1000 diluted in BSA 1%) for 2 hours at room temperature. After each step washing was done 3 times with PBS at least 3 times with shaking. Following 50 minute incubation with horseradish peroxidase-streptavidin (1 : 20000 diluted), profilin binding antibodies were detected and documented using G-BOX Chemi-Doc (Syngene, Cambridge, UK) after exposure for 5 minutes.

For cross-inhibition experiments, 150 μ l of a pooled serum consisting of 10 sera with IgE reactivity to saffron pollen, were mixed with 150 μ l of total extract of saffron pollen and shaken at 37°C for 1 hour. Incubated serum was used to assess the reactivity on PVDF membrane blotted with rCro s 2 profilin as described above.

COMPUTATIONAL ANALYSIS OF SAFFRON PROFILIN SEQUENCE

The deduced amino acid sequence of saffron profilin was blasted in GenBank within the BLAST similarity search and the percentage identities were determined by comparison of the amino acid sequences after multiple sequence alignment carried out using the BioEdit software.⁵ Further analysis to determine isoelectric pH, amino acid composition, and potential glycosylation sites of Cro s 2 protein was done by the Gene Runner software (<http://www.generunner.com>).

RESULTS

AMPLIFICATION, CLONING AND SEQUENCING OF cDNA CODING FOR Cro s 2 PROFILIN

The sequence of the newly discovered saffron profilin, Cro s 2 was deposited in GenBank under the accession number: AY898658. Amplification of saffron pollen cDNA produced a main fragment of approximately 400 bp (Fig. 1a). After cloning of this PCR product in the pET 102/D-TOPO expression vector, one clone was verified using specific primers of TOPO Kit. The PCR product showed a size of approximately 636 bp (Fig. 1b). Sequencing showed identity of sequence and fidelity of cloning (result not shown).

EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF RECOMBINANT Cro s 2

Following proliferation of the clones selected, plasmids were extracted and successfully transformed into *E. coli* strain BL21 (DE3) for expression. The ex-

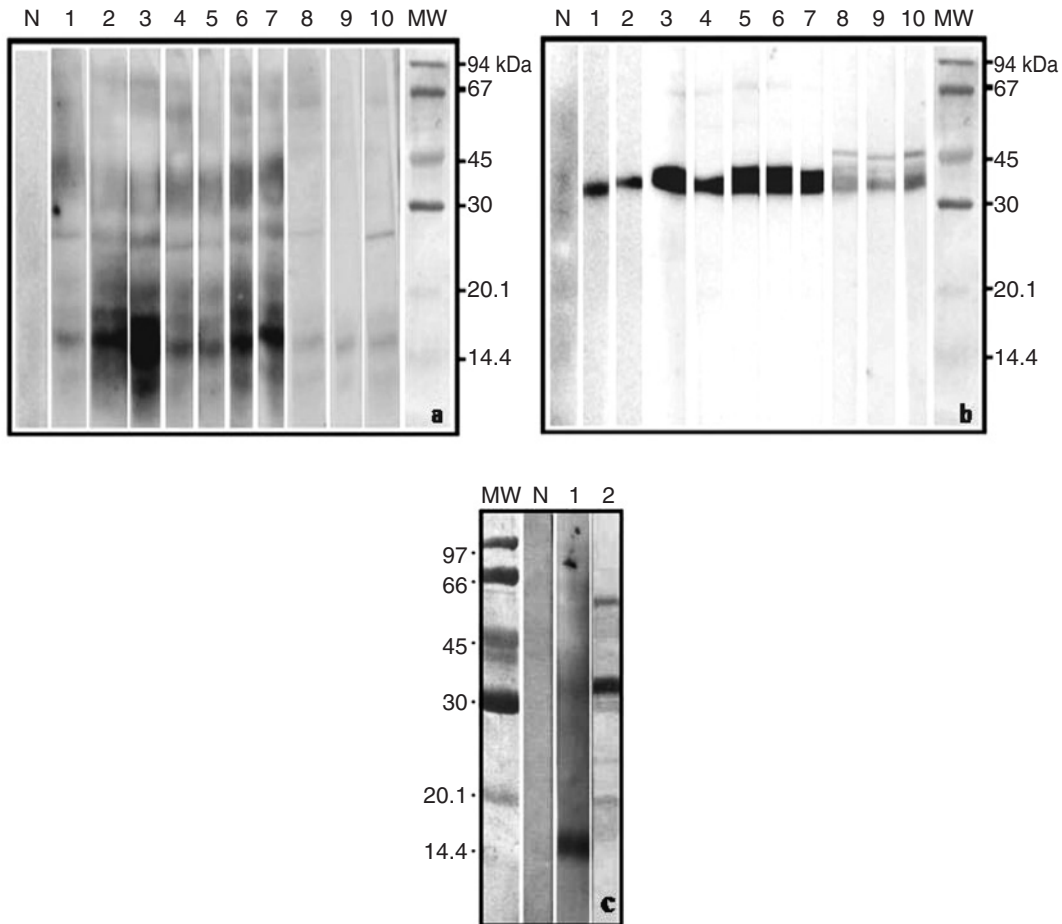


Fig. 3 Immunoblotting of natural profilin in total extract of saffron pollen and purified rCro s 2 profilin after SDS-PAGE. Lane MW: molecular weight marker, size in kDa, Lane N: non allergic sera as negative control. **a.** Lane 1-10: Immunoblotting of crude pollen extracts with individual saffron allergic sera, 14 kDa-protein bands as native profilin are seen. **b.** Lane 1-10: Immunoblotting of purified rCro s 2 profilin with individual saffron allergic sera, rCro s 2 profilin, 34 kDa protein, was recognized by profilin reactive sera from saffron allergic patients. **c.** Lane 1: Immunoblotting of crude pollen extracts with pooled saffron allergic sera, a 14 kDa- protein band as native profilin, in total extract of saffron pollen was seen. Lane 2: rCro s 2 profilin, 34 kDa protein, was recognized by pooled profilin reactive sera from saffron allergic patients.

pression of the full-length profilin region was carried out using the T7 system in which the gene of interest is expressed under the control of T7 RNA polymerase produced from the *lac* promoter. A fusion form of profilin was expressed in *E. coli* BL21 (DE3) after induction using IPTG.

SDS-PAGE analysis showed that the fusion protein was expressed mainly in an insoluble form, with a molecular weight of 34 kDa, as determined by Coomassie Brilliant Blue-stained SDS-PAGE (Fig. 2 a).

After metal precipitation, recombinant Cro s 2 profilin showed a major single band with an apparent molecular mass of 34 kDa with extra bands corresponding to the polymeric forms of the recombinant allergen. Sera from allergic patients to saffron pollen were

tested for IgE reactivity to purified recombinant profilin after SDS-PAGE (Fig. 2b).

IMMUNOBLOTTING

IgE reactivity of sera from allergic patients to saffron pollen was tested to crude pollen extracts and purified recombinant rCro s 2 profilin after SDS-PAGE. rCro s 2 profilin, 34 kDa protein, was recognized by profilin reactive sera from saffron allergic patients as well as native profilin, 14 kDa protein, in total extract of saffron pollen. These reactivities were confirmed with either individual sera or a pooled serum (Fig. 3).

The inhibition experiment showed a considerable decrease in reactivity of IgE with rCro s 2 protein after inhibition with natural profilin in total extract of saffron pollen (Fig. 4).

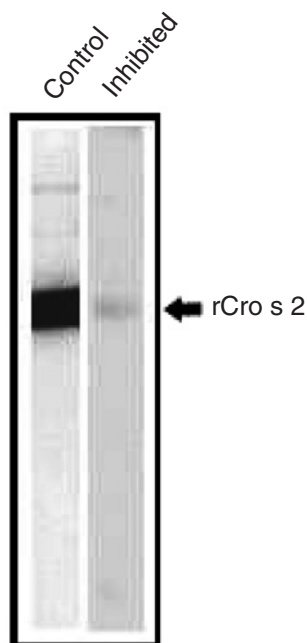


Fig. 4 IgE reactivity inhibition of pooled serum of allergic subjects to rCro s 2 with natural profilin. Blotting of rCro s 2 on PVDF membrane showed IgE reactivity of allergic sera with rCro s 2 was inhibited after incubation with natural profilin in total extract of saffron pollen.

COMPUTATIONAL ANALYSIS OF SAFFRON PROFILIN SEQUENCE

The deduced amino acid sequence of saffron profilin data was managed within the BLAST similarity search and the percentage identities were determined by comparison of the amino acid sequences after multiple sequence alignment carried out using the BioEdit software (Fig. 5).

Amino acid sequences analysis using Gene Runner of Cro s 2 showed this protein with a total of 131 amino acids contains 18 acidic, 16 basic, 74 non-polar and 57 polar amino acids has an isoelectric point of approximately pH 4.6. There was no detection of any potential glycosylation sites in Cro s 2.

DISCUSSION

Atopic diseases have been an increasing concern in recent years especially in industrialized nations.^{14,15} Allergy to pollens, pollinosis, is a well-known clinical phenomenon.^{16,17} It is known that pollen-allergic patients frequently present with allergic symptoms after sensitization to several kinds of pollens and suffer from allergic reactions, rhinitis, and bronchial asthma.¹⁸ The majority of these reactions were found to be caused by cross-reactive structures that are present in pollens referred to pan-allergens.¹⁹

Different studies in numerous plant species support the notion that profilin is an important family of

functional plant pan-allergens.^{20,21} Sequences of isolated profilin from several plants have shown high sequence identity and due to this sequence homology, a high degree of cross-reactivity can be expected with sera of allergic patients.^{7,22,23}

Profilin from plants was first isolated from birch pollen and recently profilin encoding genes from several plants have been cloned and their sequences have been identified.^{15,24-28} Saffron belongs to the Irideace family and its dried pistils and stigma is an expensive food additive and therapeutic substance. Iran is one of the most important cultivation regions for cultivation of saffron and the largest saffron producer in the world. The involvement of the saffron plant components as an allergen and the allergenicity of saffron pollen mainly in people living around saffron plantations, who were exposed to the pollen and suffered from influenza-like symptoms during the saffron flower picking season was confirmed previously.^{1,4,10} An anaphylactic reaction has been reported.³ In the Feo study the involvement of saffron components was demonstrated by RAST and skin reaction and allergenic properties of the saffron protein were analyzed by immunoblotting.² In this study only the contribution of several proteins in the IgE-binding inhibition was determined and presence of a 15.5 kDa profilin-like allergen was shown.²

In the present study, we have successfully cloned the saffron profilin and expressed the recombinant rCro s 2. The size of rCro s 2 was increased (34 kDa) greater than the natural Cro s 2 profilin due to the presence of thioredoxin at the N-terminal end and a His-tag at the C-terminal end. Purification of expressed rCro s 2 with the precipitation method has yielded a highly purified protein.

Immunoblotting assessments and inhibition of pooled serum showed a strong cross reactivity between natural saffron profilin and rCro s 2 that confirmed the presence of tags at the ends recombinant protein did not interfere with the reactivity of rCro s 2 with IgE. There have been previous reports that the presence of tags in recombinant proteins may not interfere with the activity of expressed proteins.^{22,29,30} Removal of attached tags and further analysis of reactivity has been a recent focus of study.

The purified protein was analyzed by immunoblotting after SDS-PAGE and the reactivity of sera of allergic patients was demonstrated in denaturing conditions. The presence of extra bands on SDS-PAGE gels with purified protein is related to polymerization of profilin. The oligomerization of profilin has been reported in different studies that occur with natural pollen profilins.³¹ The presence of dimer and tetramer forms of human profilin purified from platelets was shown.^{32,33}

The Cro s 2 was shown to have the highest degree of identity and similarity to *Ananas comosus*, *Corylus avellana*, *Musa acuminata* and *Corylus avellana*

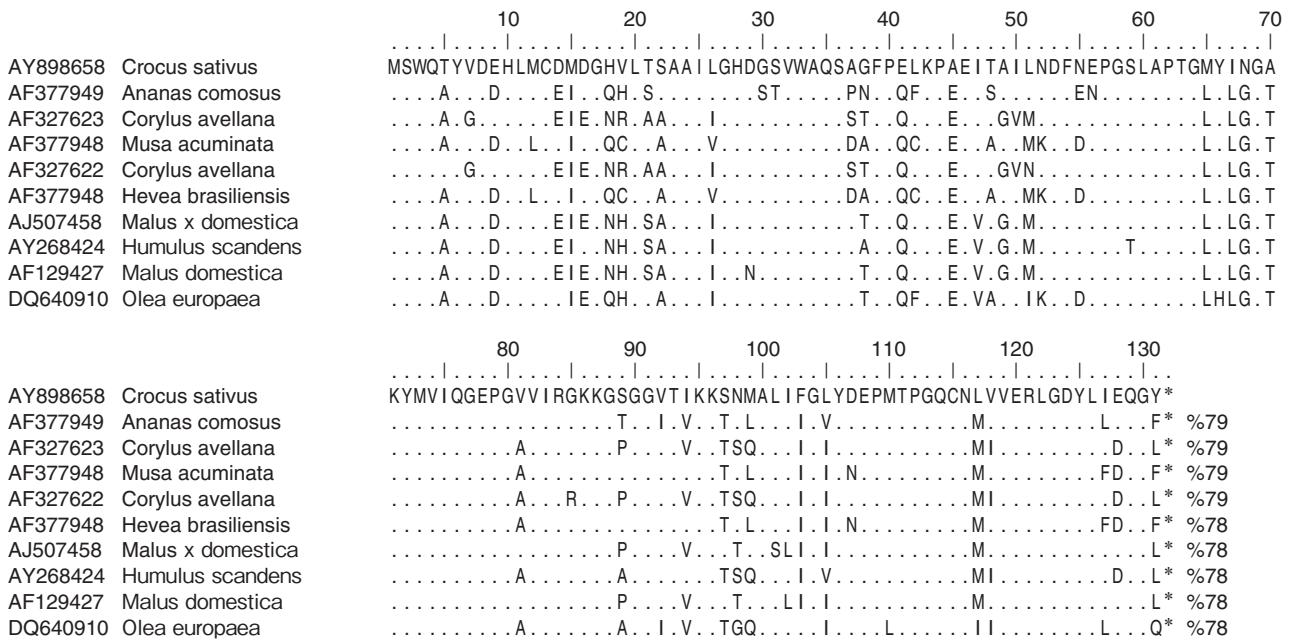


Fig. 5 Comparison of Cro s 2 sequence with different plant profilins. Amino acid sequence identity of Cro s 2 with other members of profilin family are indicated at the end of each amino acid sequence.

(79%). The calculated isoelectric point (4.6) indicated that Cro s 2 is acidic.

Several studies have shown the cross reactivity of IgE antibodies to conservative plant allergens addressing the concept that profilins are involved in these cross reactivity.^{26,34-37} Investigation of cross reactivity of rCro s 2 with other plant profilins by inhibition studies is suggested.

Techniques of genetic engineering applied to allergens have enabled the production of highly purified proteins with homogenic structures, the identification of the sequence of peptides and B cell and T cell epitopes (recombinant allergens) and allowed the better understanding of pathogenetic mechanisms of cross allergy.

The results described here may have future applications both for clinical purposes and for basic research.

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