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Differential Allergenicity of Mature and Immature Pollen Grains in Shasta Daisy (*Chrysanthemum maximum* Ramond*)*

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ABSTRACT

Weed pollen grains belonging to the *Asteraceae* family contain a variety of allergens inducing type I and IV allergies in susceptible people. The aim of this research was to compare the allergenic properties of immature and mature Shasta daisy pollen grains *(Chrysanthemum maximum* Ramond*)* to define the potential role of the maturation process on the allergenicity of *Asteraceae* pollen grains.

The immature (IP) and mature pollen (MP) grains were first studied by optical and scanning electron microscopand their protein contents were quantitatively and qualitatively analyzed. Pollen extracts were finally used to sensitize guinea pigs in order to obtain IP and MP specific antibodies. Nasal provocation tests using IP and MP crude extracts were also performed on pre-sensitized guinea pigs.

The MP extract induced IgE and eosinophilia in blood and positive skin tests in sensitized guinea pigs. Moreover, high number of eosinophils was found in the nasal mucosa of MP sensitized guinea pigs. SDS-PAGE analysis of the IP and MP protein content showed seven and five apparent bands ranging from 7 to 66kDa respectively.

According to immunoblot analysis, MP extract contained a single allergen of 66kDa. The overall results showed developmental processes of Shasta daisy pollen grains towards both morphological and molecular changes increasing their allergenic potency.

Keywords: Allergen; *Chr m 1*; *Chrysanthemum maximum* Ramond; Development; Immature pollen; Mature pollen

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INTRODUCTION

Pollen grains released from weeds belonging to the *Asteraceae* family can cause hay fever and asthma[1] and represent a major source of seasonal allergies among susceptible people in many countries.[2] The majority of weeds are wild flowers producing allergenic pollens[3] during the spring and early fall.[4,5] They are also known as occupational allergens causing asthma in the people who are frequently exposed to ornamental weed species at their working places.[6,7] *Chrysanthemum* flowers are originated from Japan and were imported to Europe some 200 years ago as ornamental plants.[8] Since then, they have been used all over the world. *Chrysanthemum* farming in Iran has become an important branch of horticulture, with huge plantations in the town of Mahallat in the Markazi province. *Chrysanthemum* flowers are now one of the most important cut flowers for export and the annual turnover is increasing every year.[9] Despite this fact, up to the present, only a few studies have been published on *Chrysanthemum* pollens. *Chrysanthemum* pollen grains have been already reported as occupational allergens by Shibata (1989) and Groenwoud *et al.* (2002).[10-12] This research project focused on pollens of *Chrysanthemum maximum* Ramond, commonly known as Shasta daisy flowers, belonging to the *Anthemideae* tribe and the *Asteraceae* family. To the best of our knowledge, the allergenicity of this species has not been investigated so far. This perennial plant is currently very common either as a wild flower in Iranian fields and mountains or as an ornamental plant in green houses (to be sold to florists and public gardens).[9] Shasta daisy flowers are grouped in a capitulum, the characteristic inflorescence of the *Asteraceae* family.[10] This capitulum has two sets of ray flowers around and disk flowers (in different sizes) from inside to outside (Figure 1.a, b). In this research project we studied both intrastructural and molecular properties of *Ch. maximum* pollen grains in different temporal maturation steps. The allergenicity of the immature and mature pollen grains was also investigated to determine whether growth processes and ontogeny of the pollen grains influence their allergenic potency.

MATERIALS AND METHODS

Sampling

Shasta daisy flowers were purchased from plant and flower markets of Tehran city at the beginning and at the end of the flowering seasons (May 2007-September 2007). Fresh immature and mature pollen grains were selected by passage through mesh sieve with pores of 25 µm in diameter. Purity of both samples was checked by optical and scanning electron microscopies (SEM). Samples were briefly dried and frozen at -20°C until used.

Figure 1: *Ch. maximum* flowers have Capitule Inflorescence (yellow): Ray flowers (Rf) and Disk flowers (Df). a) *Ch. maximum* in early flowering season and b) *Ch. maximum* at the end flowering season

Preparation of Semi-Thin Sections of Pollen Grains

In order to study the ultrastructure of pollen grains, semi-thin sections of mature and immature pollen grains were prepared. After fixation of samples by glutaraldehyde solution, complementary fixation with osmium in phosphate buffer saline (PBS) (0.2M, PH 7.4), dehydrating with acetone and propylene oxide were performed. Samples were then saturated with 48gr resin, 19gr decenyl succinic anhydride, 33gr acid methyl anhydride and 2gr 2,4,6-tri dimethyl amino methyl phenol.[13] Semi thin sections were prepared by the use of ultra microtome (Leica, Germany). After staining with toluidine blue, the sections were observed under optical microscope.

Scanning Electron Microscopy (SEM)

SEM was used in order to study the finer structure of mature and immature pollen grains of Shasta daisy flowers. Samples were dried prior to gold coating and were then observed by the SEM electron microscopy (XL 30, Philips, Holland) and were scanned.[14,15]

Preparation of Pollen Extracts

For the preparation of pollen protein extracts, an equal amount of each sample, 50mg per ml (1:20 w:v), was suspended in 0.01M PBS buffer pH7.4 for 18 hours at 4°C under stirring and then centrifuged at 10,000g for 40 minutes at 4°C. The supernatant was collected and dialyzed against PBS buffer overnight.[14]

Protein Studies and Profiling by SDS-PAGE

The protein concentration of IP and MP PBS extracts was measured by Bradford protein assay method using bovine serum albumin (BSA) as standard.[16] SDS-PAGE was carried out by vertical discontinuous gel electrophoresis method.[14] Extracted proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (90µl of each pollen extract per well) and detected by Coomassie blue staining. Isolated protein bands were then analyzed by photo EP software densitometry (photo EP version 5.50, Amir Kabir Bio.Medical EngGroup, Hoshmand Fanavar Co, Tehran, Iran).[17]

Experimental Animals

Male guinea pigs (Hartly) (4-6 weeks of age) weighting about 350 gr were purchased from the Pasture Institute of Iran. The animals were housed in an air-conditioned room at 25 ± 2°C with an 8h light/8h dark condition. They were fed with standard laboratory diet (water and carrot with additional vitamin C).[15] the animals were then divided into four groups of seven animals each. The first group was immunized IP with PBS extracts, the second group with MP PBS extracts. The immunization dosage consisted of 50µg protein diluted in 50 µl 0.05M PBS with 50µg Alum. This dose was repeated without adjuvant on days 10 and 20 after the first injection to reach a total of three injections per month.[18] The third one with PBS only and finally the sham group (without injecting any proteins or PBS).

Eosinophil count in Blood and Serological Tests

Blood samples were drawn directly from the hearts.[6,18] Blood samples were collected from guinea pigs 8 hours, 48 hours and 7 days after the last injection. Eosinophils in blood samples were counted by sysmex (KX21). Total IgE was measured in guinea pigs sera by a standard Elisa kit (serotec, UK).[18]

Skin Test

One week after the last sensitization, each animal was intradermally (ID) skin tested on the abdomen with 25µl of the crude extract (containing 5µg of protein in PBS pH 7.4). The PBS and histamine acid phosphate were also intradermally injected as negative and positive controls, respectively.[19,20] The skin reactions on abdomens were read at 30, 60 and 120 minutes after ID injections and each reaction was quantified on the basis of the wheal diameters. Wheal diameters of more than 6 mm were considered positive.[20]

Nasal Provocation Test (NPT)

For NPT, pollen protein extracts (3µg of protein in 5µl PBS) were applied into the nasal mucosa of each animal using a dropper.[21] For the control group, only PBS buffer was used. Nasal clogging and the number of sneezes were the criteria to assess the intensity of allergenicity of different extracts. After 8 hours, a direct slide was also prepared from the secretions of the nostrils of the guinea pigs by a sterile swab .The samples were then stained with Wright dye solution. Presence of more than 5 eosinophils in each microscopic field of the nasal secretions was considered as positive.

Western Immunoblotting

After electrophoresis, proteins were transferred from the gel on to PolyVinylene Di Fluoride (PVDF)

membrane (0.45µ, SIGMA). The membranes were blocked for one hour at room temperature with 0.05% Tween20 and 3% nonfat milk in PBS.[14] The membrane was washed three times for 10 min in PBS-Tween20 and incubated overnight with 1:15 diluted serum of allergic subjects. After the incubation, the membrane was washed and incubated for one hour with 1:7000 dilution of anti-guinea pig IgG_1 conjugate (Serotec, UK) with horse radish peroxidase in PBS.[22-24] The membrane was washed again and then treated by Electrogenerated Chemiluminescence (ECL) method (Amersham Bioscience, USA). The specific IgE reactive bands were revealed after 1min contact with X-OMAT Kodak Film.[14]

Statistical Analysis

Statistical analysis was performed with SPSS version 11 software using the one-way ANOVA, followed by Duncan multiple-range test. *P*values<0.05 were considered significant.

RESULTS

Optical microscopy of pollen semi-thin slices revealed several dissimilarities of exine surfaces between IP and MP (Figure 2.a, b).

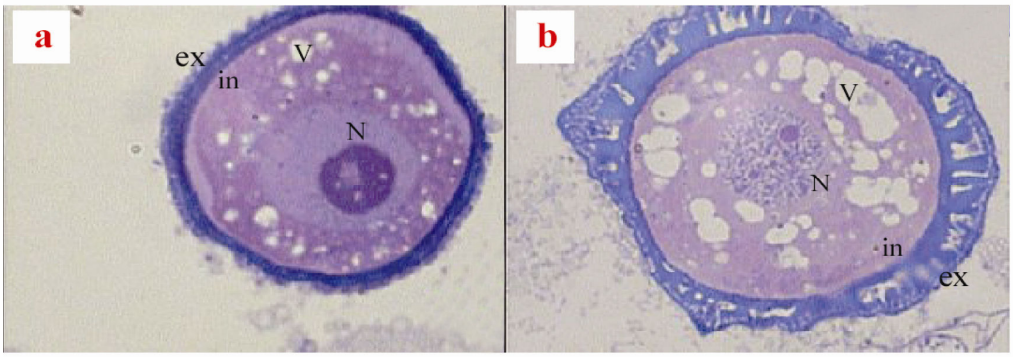

Figure 2. Semi-thin sections of *Ch. maximum* pollen grains; a) Immature pollen (IP) collected at the beginning of the flowering season; b) Mature pollen (MP) at the end of the flowering season (magnified Xl00).
(ex= exine, in= intine, V=vacuole, N= Nucleus)

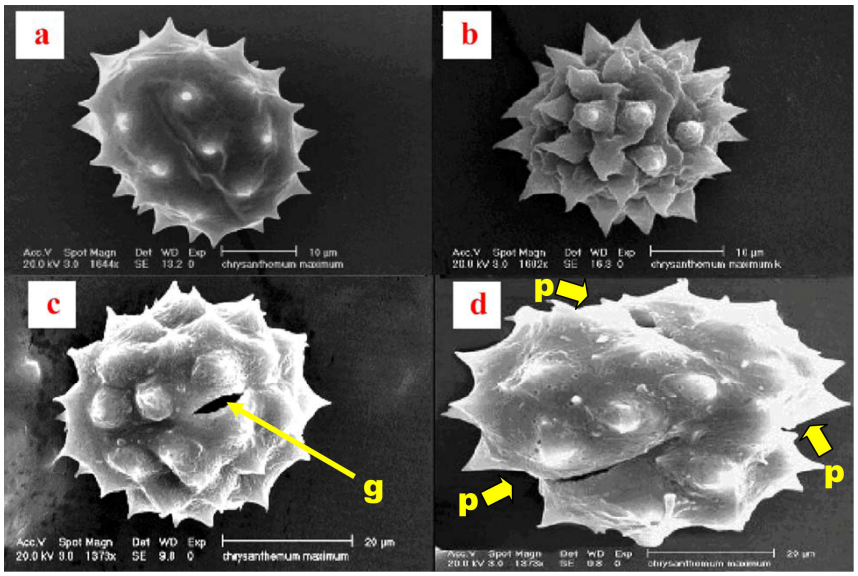

Figure 3. SEM micrographs of *Ch. maximum* pollens in different developmental stages; a) Immature pollen grains(IP) magnified 1644x; b-d) Mature pollen grains(MP), magnified 1602x(b), magnified 1373x(c,d) (g=groove, p=pores).

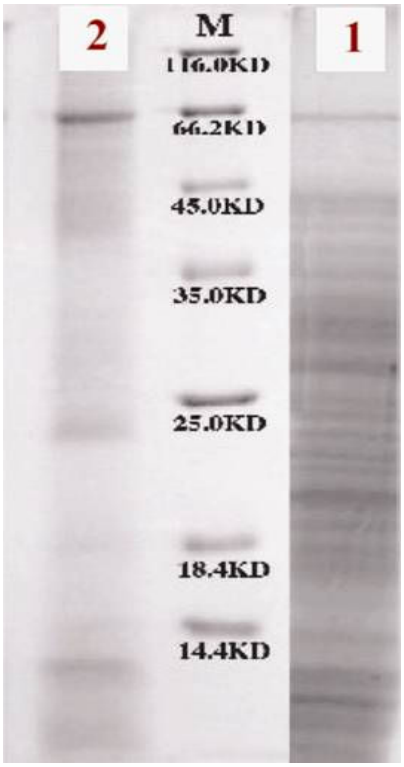

Figure 4. Image of the stained SDS-PAGE gel showing protein profiles of the IP and MP PBS extracts in *Ch. maximum*. M: markers, lane 1: IP and lane 2: MP protein extracts.

The SEM micrographs of, *Ch. maximum* pollen grains showed that IP were oval, with thorny exine on their surfaces, whereas MP displayed grooves and pores with more roughness of the exine surface due to the presence of tough thorns (Tri-colporate form) (Figure 3.a-d).

Bradford protein assay revealed that the total protein content of IP was higher than that of MP. The protein concentrations of IP and MP extracts were 353.3 and 340.0µg/ml, respectively.

SDS-PAGE separations of IP protein extracts revealed different protein fractions at: 7, 14, 18, 25, 35, 45 and 66kDa (Figure 4.1). MP extracts exhibited fewer protein bands with relative molecular masses of 7, 18, 21, 40, and 66kDa (Figure 4.2). Densitometric analysis of the major protein bands brought to light noticeable differences between IP and MP in terms of protein distributions and the levels of expression for different Coomassie-stained proteins (Figure 5.a, b). Although the 66kDa protein band was the major component of MP but the minor component for IP protein extracts, its concentration increased through the maturation process from about 21.8% in IP (Figure 5.a) to 34.9% in MP total protein content (Figure 5.b).

Results of guinea pigs sensitization using revealed that the blood eosinophil count was significantly higher (P=0.024) in animals sensitized by MP (12 ± 0.9 after 8 h) compared to the IP sensitized group (8±0.5 after 8h) (Table 2). The level of total serum IgE also significantly increased in MP sensitized group in comparison to that obtained from sera of guinea pigs treated by IP (about three time higher after 8h, (P=0.007) (Tables 2). Skin tests performed in the same conditions induced wheals on both IP and MP sensitized animal skins.

Table 1. Immunological results of immature and mature pollen grains

Parameters	Immature pollens	Mature pollens	*P* value
Wheal diameter (mm) Mean±SD	5±0.7	10±0.2	P=0.002
Eosinophils from NPT 40X(0.0625mm^2) Mean±SD	2.5±0.7	7.8±2.6	P=0.01

Table 2. Kinetics of blood eosinophils and total serum IgE in guinea pigs after immunization with immature and mature pollen grain extracts

	Immature pollens			Mature pollens			Control group		
Time after immunization	8h	48h	1wk	8h	48h	1wk	8h	48h	1wk
Eosinophils (× 10^4 cells/ml) Mean±SD	8±0.5	4.5±1.3	4±0.9	12±0.9	9.5±2.2	6±0.5	4.5±1.9	5.5±0.2	3.1±0.7
Total IgE (µg/ml) Mean±SD	4±0.7	6±0.9	3±2.2	12 ±0.2	10±0.00	8 ±0.5	3.9±1.3	5.5±0.5	2.5±0.9

However, after 30 and 120min, wheal diameters were significantly larger in MP sensitized animals compared to the group treated with IP (P<0.05). Maximum wheal diameters in IP and MP pre-sensitized animals were 10mm and 14mm respectively, while wheal diameters of positive controls did not exceed 9mm (maximum of 4.5mm in negative controls) (Table 1).

NPTs caused nasal itching, frequent sneezing, and nasal clogging after 30 to 120min of their administrations into the nose of both IP and MP sensitized animals. Nevertheless, there was a three-fold increase in the number of eosinophils for MP sensitized group (7.8±2.6) compared to animals treated with IP (2.5±0.7) (Table 1).

Immunoblot analysis using sera of sensitized guinea pigs revealed a high specific IgE reactivity to the 66kDa protein band present in the MP crude extract of *Ch. maximum.* Similar band was not observed for IP crude protein extract. This allergen, which is identified for the first time to the best of our knowledge, was named"Chrm1" based on the regulations of allergen's terminology (Figure 6).

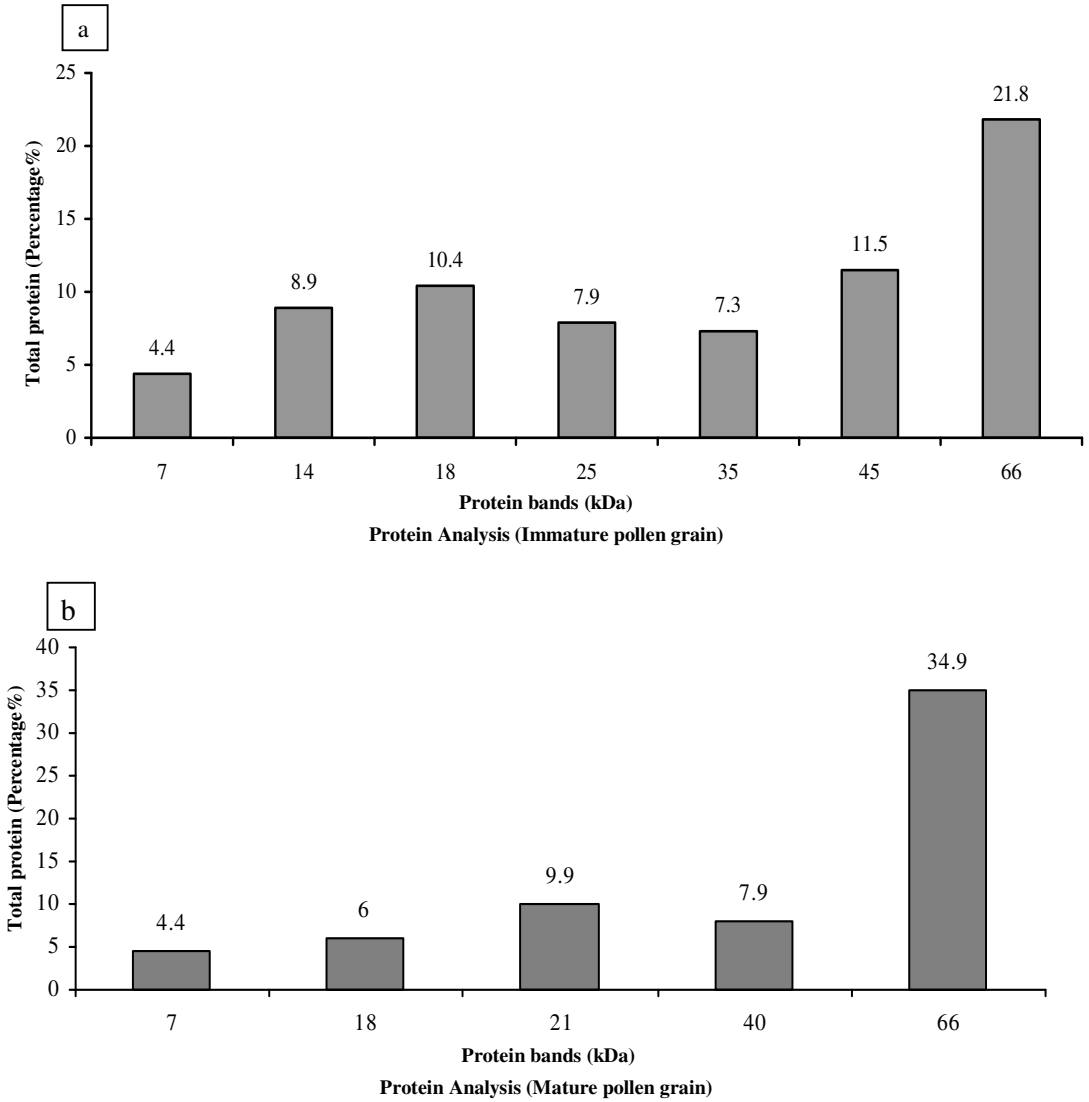

Figure 5. Densitometry analysis of SDS-PAGE protein profiles: a) IP protein extracts including proteins with relative molecular masses of 7, 14, 18, 25, 35, 45 and 66kDa and b) MP protein extracts with 5 major proteins at 7, 18, 21, 40 and 66kDa.

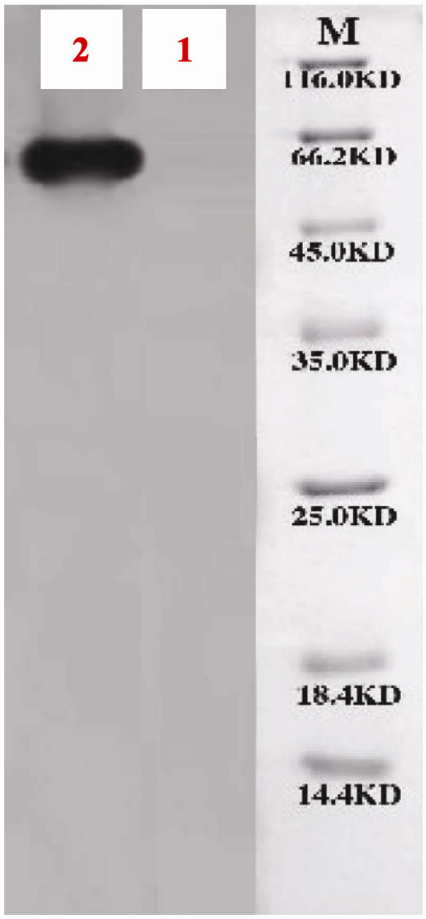

Figure 6. Comparative immunoblots probed with sera from IP (lane 1) and MP (lane 2) sensitized guinea pigs. M indicates the standard marker.Immunoblots showed the major protein of 66kDa "*Chrm1*"in the mature pollen extract of *ch. maximum*.

DISCUSSION

Chrysanthemum spp. flowers are now one of the most important cut flowers for export, and the annual turnover is growing every year.[10,11] At the same time, however, the number of allergic complaints also appear to have increased among some people.[10] Comparative morphological and structural studies of *Ch. maximum* IP and MP revealed noticeable differences regarding their pollen walls permeability. MP exhibits many vents and pores through the exine while these structures are totally lacking in IP. These vents represent a direct passage across the resistant pollen wall for discharging the grain contents into the surrounding environment.[1] This feature facilitates the liberation of MP allergenic contents.[11] Both IP and MP have uneven and very rough surfaces which can allow a better adhesion of these grains on the mucosa of respiratory airways (Figure 3.a-d).

In the present study, various tests were used to evaluate the allergenicity and immunogenicity of *Ch. maximum* (IP and MP).[25] These tests included skin tests, eosinophil count in nasal secretions and peripheral blood and measuring total IgE in sera of IP and MP sensitized guinea pigs. The results of all these comparative tests indicated that the allergic potential of MP was higher than IP.However, the difference in the amounts of proteins in these two pollen grains was not statistically significant. The present study showed that other structural and molecular factors may bring about differences between IP and MP allergenicity (Figure 3). Therefore, besides total protein contents, the level of expression and the conformation of individual protein components have to be taken into consideration. Results of this study are of special interest and dose-response test methods using individual major allergens[25] could be proposed in order to recommend safe exposure levels for pollens.

Despite the lack of visible IgE-binding proteins in immunoblots probed with sera from IP sensitized animals, according to skin test and NPT results, Shasta daisy IP PBS extracts were also able to induce allergic reaction, although at lower levels, in sensitized guinea pigs. It could be due to lower amount of this particular antigen in IP. This fact reflects the lower sensitivity of our in vitro immunoblot analysis compared to in vivo tests. Moreover, both IP and MP sensitized groups displayed higher total IgE compared to control groups.

In conclusion, both IP and MP of Shasta daisy flowers were able to induce allergic reaction. However, an increasing allergenicity has been observed in MP which could be directly related to the higher expression of a 66kDa allergen. Further analyses are needed to unravel the nature of this novel IgE-binding protein**.** Horticultural workers and florists must take safety precautions to minimize their exposure to Shasta daisy pollen grains.

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