

Impact of traffic-related air pollution on the expression of *Platanus orientalis* pollen allergens

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Abstract Air pollutants and their interaction with environmental allergens have been considered as an important reason for the recent increase in the prevalence of allergic diseases. The aim of this study was to investigate the traffic pollution effect, as a stressor, on *Platanus orientalis* pollen allergens messenger RNA (mRNA) and protein expression. *P. orientalis* pollen grains were collected along main streets of heavy traffic and from unpolluted sites in Mashhad city, in northeast Iran. The pollen samples were examined by scanning electron microscopy. To assess the abundance of pollen allergens (Pla or 1, Pla or 2, and Pla or 3) from polluted and unpolluted sites, immunoblotting was performed. Moreover, the sequences encoding *P. orientalis* allergens were amplified using real-time PCR. Scanning electron microscopy showed a number of particles of 150–550 nm on the surface of pollen from polluted sites. Also, protein and gene expression levels of Pla or 1 and Pla or 3 were considerably greater in pollen samples from highly polluted areas than in pollen from unpolluted areas ($p < 0.05$). In contrast, no statically significant difference in Pla or 2 protein and mRNA expression level was found between samples from the two areas. We found greater expression of allergens involved in plant defense mechanisms (Pla or 1 and Pla or 3) in polluted sites than in

unpolluted ones. The high expression of these proteins can lead to an increase in the prevalence of allergic diseases. These findings suggest the necessity of supporting public policies aimed at controlling traffic pollution to improve air quality and prevent the subsequent clinical outcomes and new cases of asthma.

Keywords mRNA level · Pathogenesis-related protein · *Platanus orientalis* · Traffic pollution

Abbreviations

IUIS International Union of Immunological Societies
PR Pathogenesis-related
SEM Scanning electron microscopy

Introduction

The prevalence of allergic diseases has risen in recent decades (Asher et al. 2006; Kim et al. 2013; Huang et al. 2015), especially in industrialized countries (Armentia et al. 2002; D’Amato et al. 2010; Ghiani et al. 2012). An important reason for this increase may be air pollution. Some studies indicate that living in urban areas and near high-traffic roads are related to acute and chronic respiratory diseases (Salvi 2007; D’Amato et al. 2010).

Pollen is considered as important airborne allergens in the environment (Bosch-Cano et al. 2011; Ghiani et al. 2012). *Platanus orientalis* is the most common ornamental plant widely cultivated along streets and in parks of cities in southwest Asia, southeast Europe, and the USA (Sankian et al. 2012).

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Three major allergens have been identified in *P. orientalis*: Pla or 1, a non-glycosylated protein, and a member of invertase/pectin methylesterase inhibitor family, with a molecular weight of 18 kDa; Pla or 2, a 43 kDa glycoprotein, and a polygalacturonase; and Pla or 3, belonging to non-specific lipid-transfer proteins (ns-LTPs), and a 9–10 kDa protein (Sankian et al. 2012). In a study on 19 Iranian *P. orientalis*-allergic patients, IgE-binding prevalence to Pla or 1, Pla or 2, and Pla or 3 was identified 21, 27, and 27 %, respectively (Pazouki et al. 2008).

According to the literature, air pollutants can affect pollen grains in several ways; they can damage cell wall structure, resulting in increased release of allergenic proteins (Chehregani et al. 2004; Motta et al. 2006; Ghiani et al. 2012), bind with pollen grains and act as adjuvants (Cortegano et al. 2004), and finally, upregulate the expression of some allergens, resulting in increasing allergic reactions in these areas (Cortegano et al. 2004; Suárez-Cervera et al. 2008; Aina et al. 2010).

Several studies have focused on the possible effects of the interaction between air pollutants and plant pollen. Bosch-Cano et al. (2011) showed that air pollutants can induce a large amount of allergenic pollen in some ornamental trees, providing new sources for aeroallergens. In addition, Armentia et al. (2002) reported greater protein content and pollen allergenicity in urban areas than in rural areas. This group suggested that this finding explains the high prevalence of allergy in urban areas and should be considered in diagnosis and specific immunotherapy. In another laboratory-based experiment, Lu et al. (2014) showed that pollutant gases could be found on the pollen surface, increasing the allergenicity and release of allergenic proteins in oriental pollen.

These findings propose an undesirable effect of air pollution on airborne allergens. However, few studies have compared pollen allergen expression quantitatively in highly polluted and rural areas (Suárez-Cervera et al. 2008; Bryce et al. 2010; Cortegano et al. 2004; Armentia et al. 2002; Behrendt et al. 1999), and no research has investigated the expression of these proteins at the messenger RNA (mRNA) level. In this work, we investigated the traffic pollution effect, as a stressor, on *P. orientalis* pollen allergens mRNA and protein expression. We selected *P. orientalis* as a model because exposure to *Platanus* pollen is an important contributor to allergic symptoms during the pollination period in March and April (Lu et al. 2014). Also, *Platanus* is widely cultivated in streets and parks around the world (Pourkhabbaz et al. 2010), including the area of our study, Mashhad (Sankian et al. 2012), which is among the main polluted cities in Iran (Azizi 2011). In addition, since motor vehicle exhaust emissions are a major source of air pollution in developing countries, we chose traffic as the source of pollution (Azizi 2011).

Methods and materials

Study area and meteorological conditions

This study was conducted in Mashhad, the second most populous city in Iran and capital of Razavi Khorasan Province. Mashhad, with a population of 2,749,374 (Statistical Center of Iran, 2011 Census website), is located in northeast Iran in the Kashaf River valley, between the Binalood and Hezar-masjed mountain ranges.

Pollen samples were collected from *P. orientalis* trees during the spring pollination in April of 2013 and repeated in 2014 to compare with the 2013 results. Sampling and storage procedures were the same for all samples.

Three polluted and two unpolluted areas (Fig. 1) were selected based on their population densities (recommended by the Statistical Center of Iran) and the average number of motor vehicles per day on the main streets according to data provided by Mashhad Traffic and Transportation Organization (Table 1). The pollen grains were collected from four sites of each selected area (three different trees of each site) and then pooled. Samples from polluted sites were collected near heavy-traffic and highly populated streets within 10 m from the traffic source, and samples from unpolluted sites were collected in vegetated sites at least 500 m from motor vehicle traffic. Pollen samples were isolated by passage through mesh and their purity was determined using light microscopy. All samples were more than 98 % pure. Annual mean, maximum and minimum air temperatures, and total rainfall (2013–2014) were recorded using one standard meteorological station located in Torogh Dam (Table 2, Fig. 1). Data were provided by Khorasan Razavi Regional Water Authority and Meteorological Office (Table 2).

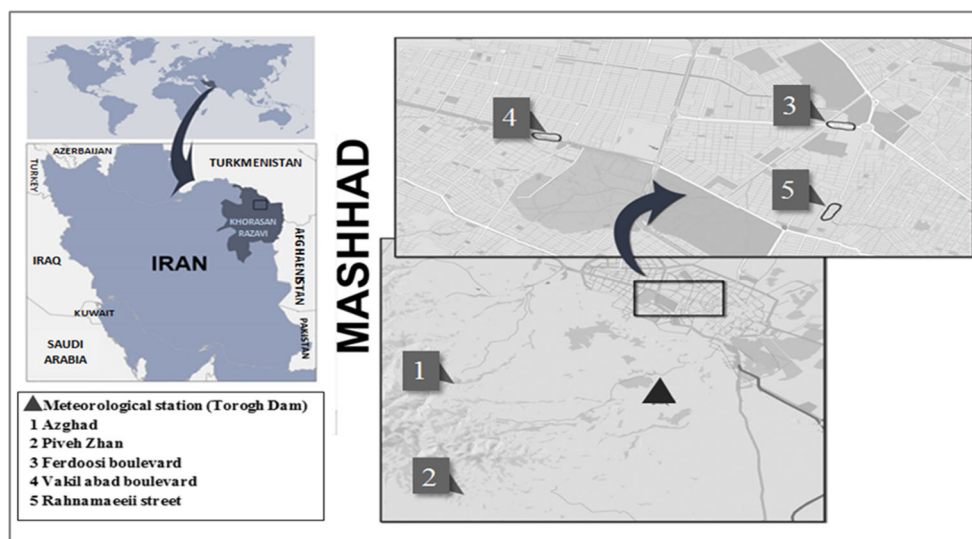
Scanning electron microscopy analysis

Pollen grains were placed on double-sided carbon tape and coated with gold. The coated samples were examined by scanning electron microscopy (VEGA/TESCAN; Czech Republic) using the following parameters: voltage, 10 kV; resolution, 10 nm; and working distance, 3.892 mm.

Expression of *P. orientalis* recombinant allergens and production of polyclonal antibody

Three recombinant allergens of *P. orientalis* (Pla or 1, Pla or 2, and Pla or 3) were expressed in *Escherichia coli* BL21-CodonPlus cells. Bacterial cells were harvested by centrifugation at 3000×g for 15 min at 4 °C, treated with lysis buffer, sonicated, and pelleted by centrifugation at 10,000×g for 10 min at 4 °C; the supernatant containing the expressed allergens was then purified by metal-affinity chromatography on a Ni-IDA column (Pars Tous Biotechnology, Iran).

Fig. 1 Map of the study sites in Mashhad city, capital of Razavi Khorasan Province; five sample collection areas were selected. The areas were categorized into two groups (polluted and unpolluted) based on the number of motor vehicles on the streets per day. Meteorological station was located at Torogh Dam, situated 15.41 km of Vakil abad, 16.40 km of Ferdoosi, 14.46 km of Rahnamaeeii, 17.53 km of Azghad, and 20.56 km of Piveh Zhan



Ten to 16-week-old female rabbits (2.5–3.0 kg weight) purchased from the Razi Vaccine and Serum Research Institute were housed and fed with standard laboratory methods. Polyclonal antibodies were raised against purified rPlA or 1, rPlA or 2, and rPlA or 3. Animals were sensitized by injection of 100 µg of polyacrylamide gel homogenate containing each recombinant allergen. Injections were repeated four times, for 6 weeks, with 2 weeks intervals. Antibody production was confirmed by immunoblotting with injected allergens and pre-immune rabbit serum as a control. Polyclonal antibodies were purified using protein A affinity chromatography (Amersham Pharmacia Biotech).

Preparation of pollen extracts

Protein extracts were prepared from pollen grains according to a previously described method (Subiza et al. 1989). Three milligrams of each sample were weighed and defatted in acetone. After drying, pollen proteins were extracted in 0.01 M phosphate-buffered saline (PBS) pH 7.4, for 16 h at 4 °C with

stirring. The suspensions were then centrifuged at 14,000×g for 1 h at 4 °C and the supernatants were dialyzed against PBS. Total protein contents were measured according to the Bradford method (Bradford 1976). The extracts were diluted with distilled water to lie within the calibration range. Two hundred microliters Bradford reagent was added to 800 µl of each extract, and the absorbance was measured at 595 nm. The use of bovine serum albumin (BSA) as the standard enabled the total protein content of the pollen extracts to be quantified. The extracts were then stored at −20 °C until use.

SDS-PAGE and immunoblotting for assessment of protein production from pollen extracts

To compare the amount of pollen allergens in extracts from polluted and unpolluted sites, extracted proteins were separated by SDS-PAGE (Laemmli 1970) and detected by Coomassie Blue staining. Immunoblotting was then performed according to Aina et al. (2010). Briefly, equal amounts of total protein extracts prepared from pollen from polluted

Table 1 Geographic location and mean number of vehicle per day in selected areas during 2013 and 2014

Collection site		Coordinates	Altitude (m)	Mean number of vehicle per day	
				2013	2014
Polluted areas	Ferdoosi	36°19' 25.45" N 59°34' 29.63" E	1003.45	9515	10,582
	Vakil abad	36°19' 2.96" N 59°32' 2.65" E	1003.95	36,106	26,724
	Rahnamaeeii	36°18' 25.98" N 59°34' 23.26" E	998.78	25,082	25,984
Unpolluted areas	Piveh Zhan	36°03' 23" N 59°20' 46" E	1798	0	0
	Azghad	36°11' 57.0" N 59°20' 14.0" E	1788	0	0

Table 2 Climate variables of selected areas during 2013 and 2014

Meteorological variables	2013	2014
Mean annual temperature (°C)	14.7	14.3
Annual maximum temperature (°C)	22.7	22.1
Annual minimum temperature (°C)	6.6	6.4
Total annual rainfall (mm)	234.2	203.5

and unpolluted areas were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore Corp., Bedford, MA, USA). After blocking with 2 % BSA, the extracts were exposed to specific affinity-purified polyclonal antibodies (diluted 1:2000 in PBS) for 3 h at room temperature. After the incubation, the extracts were incubated with goat anti-rabbit IgG conjugated with peroxidase (diluted 1:20,000) (Bio-Rad, CA, USA) for 1 h at room temperature. The lanes were washed and visualized using an ECL detection kit (GE Healthcare, Buckinghamshire, UK). The reactions were revealed and documented by Gel-Doc (Syngene, Cambridge, UK).

RNA extraction

Total RNA was extracted from *P. orientalis* pollen by a modified guanidinium isothiocyanate method (Chomczyński et al. 1968). The pellet was washed using 70 % ethanol in DEPC-treated water, air-dried, and dissolved in 30 µl of DEPC-treated water. The extracted sample was then treated to DNase and purified by the phenol-chloroform method. The quality of the purified RNA was evaluated by gel electrophoresis on a 1 % agarose gel. The RNA concentration and purity were determined by spectrophotometry at 260 and 280 nm (A 260/A 280).

cDNA synthesis and real-time PCR amplification of allergens

Complementary (cDNA) was synthesized from 2 µg of total RNA from each sample using a cDNA synthesis kit (Fermentas, Lithuania) with a random hexamer as the primer. Forward and reverse primers for sequences encoding Pla or 1, Pla or 2, and Pla or 3 were designed using Beacon software (Applied Biosystems, USA). Pla or 1 forward primer was 5' CCTAAGAGCCACAGTGC GGAT3' and reverse primer was 5' TTTCAAGATGCGACCAATAAATGTTTG3'; Pla or 2 forward primer was 5' AAGGACAGAAGGCTTGGGCT3' and reverse primer was 5' CGGCGTTCTTGAGGAAGTTGA3'; Pla or 3 forward primer was 5' GACGCTAAAACCAACCCAGA3' and reverse primer was 5' TTA CTTCACCTTGAGCAGTCAA3'. The sequences encoding Pla or 1, Pla or 2, and Pla or 3 were then amplified using specific primers and SYBR® Green real-time PCR Master

Mix (Pars Tous biotechnology, Iran) in a Rotor Gene 6000 Multiplex System (Rotor Gene-Q, Qiagen, Germany). The reaction mix contained 2 µl of cDNA (250–400 ng), 1 µl each of the forward and reverse primers (100 pmol), 10 µl of 2× SYBR Green mix, 0.4 µl of ROX, and dH₂O to bring the volume to 25 µl. The optimized parameters used for the thermocycler were the following: short hot-start at 95 °C for 10 min, followed by 40 cycles, each consisting of denaturing at 95 °C for 20 s, annealing at 60 °C for 30 s, and extension at 72 °C for 20 s. The 2013 samples were run in triplicate and 2014 samples in six replicates. As the final step of real-time PCR, melting curves were incorporated from 68 to 78 °C rising by 1°. Data analysis was performed using Rotor Gene 6000 Series Software 1.7. Beta-actin was used to normalize expression. Finally, the fold difference of expression in samples from polluted and unpolluted areas was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001).

Statistical analysis

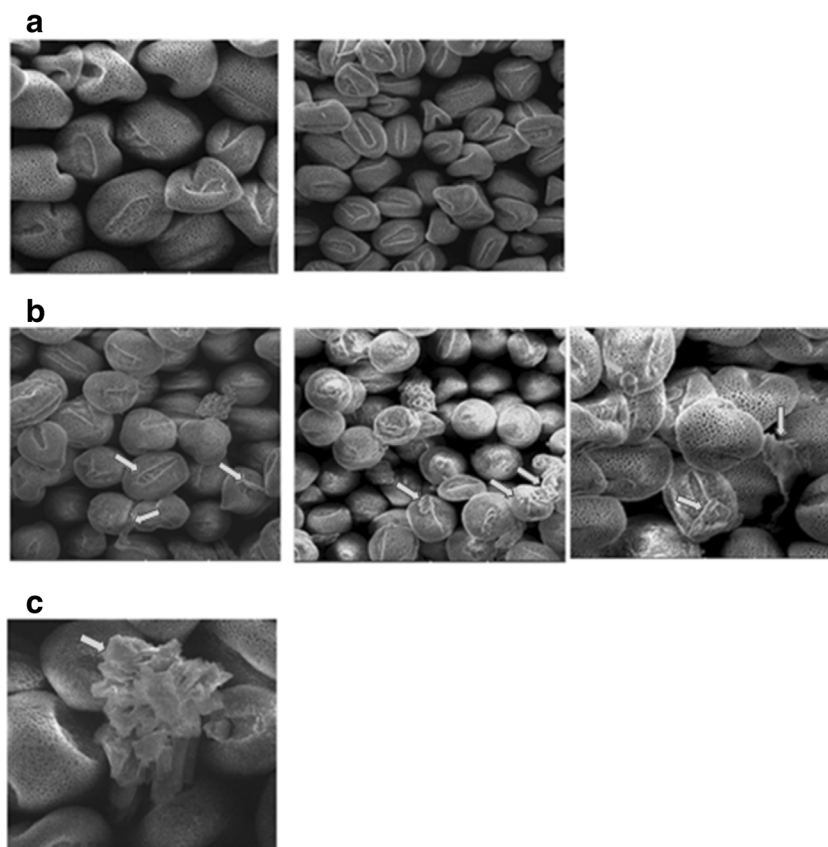
Results were represented as the mean ± SD (standard deviation). Statistical analysis was performed using GraphPad Prism (GraphPad Software). If not following Gaussian distribution, as tested by D'Agostino's normality test, data were logarithmically transformed before statistical analysis. Unpaired Student's *t* test was used to statistically evaluate differences in Pla or 1, Pla or 2, or Pla or 3 expression levels between pollen collected from polluted and unpolluted areas, and between the years 2013 and 2014. To compare the expression levels between different polluted areas (Ferdoosi, Vakil abad, Rahnamaeei), One-way analysis of variance (ANOVA) test was applied. When revealing significant differences, post hoc pairwise multiple comparison procedures were performed using Tukey post hoc test. Differences were considered statistically significant when the *p* value was <0.05.

Results

Microscopic characterization of pollen structure

Results of SEM showed that *P. orientalis* pollen grains are prolate, subprolate, spheroidal, or subspheroidal with trenches on their surfaces and a diameter of 18–25 µm. Further analysis revealed a number of particles ranging from 150 to 550 nm on the surface of pollen from polluted areas. We investigated pollen samples of all areas to estimate the percentage of pollen with the particles on its surface. The sub-micronic particles were observed on 25–70 % of pollen from polluted samples (Fig. 2b) but only on 0–9 % of pollen from the unpolluted samples (Fig. 2a).

Fig. 2 Microscopic images of the *Platanus orientalis* pollen. **a** Pollen collected from unpolluted areas. **b** Pollen collected from traffic polluted areas. Particles (arrows) can be seen on its surface. **c** Aggregation of particles (arrow) on the surface of pollen gathered from polluted areas



Assessment of protein production from pollen extracts using SDS-PAGE and immunoblotting

Immunoblotting was performed to compare the amount of allergenic proteins in pollen samples collected from polluted and unpolluted sites. Figure 3 shows a representative membrane of allergens probed with specific antibodies. Immunoblotting revealed that Pla or 3 expression was considerably greater in pollen samples from highly polluted areas than in samples from unpolluted areas. In contrast, no detectable difference was found between the levels of Pla or 1 and Pla or 2 proteins in pollen collected from all areas.

Relative quantification of expression of *P. orientalis* genes by real-time PCR

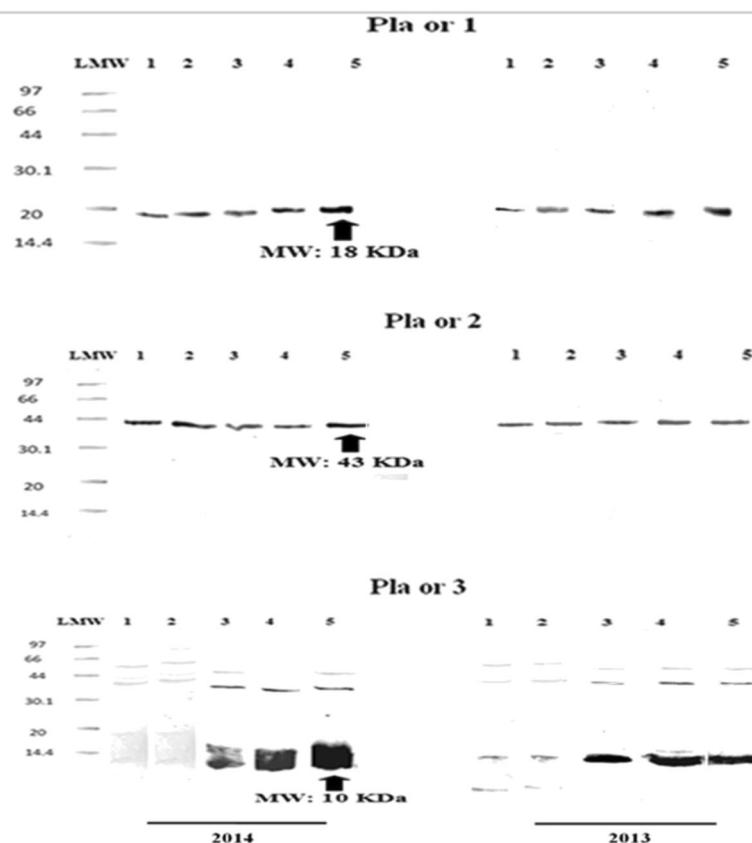
The relative quantitative expression for each gene was calculated and statistically analyzed. As shown in Fig. 4, the expression of Pla or 3 mRNA was significantly greater in polluted areas than in unpolluted areas ($p < 0.0001$). In addition, Pla or 1 mRNA expression was significantly greater in pollen collected from polluted areas than in those from unpolluted areas ($p < 0.0001$). No statistically significant difference was found in the expression of Pla or 2 mRNA in samples from polluted and unpolluted areas (Fig. 4).

Discussion

Environmental pollution has been considered as a possible explanation for the recent increase in asthma and allergic diseases (Azizi 2011; North et al. 2014). Published data suggest that air pollutants can exacerbate allergic reactions by their interaction with pollen grains (Ghiani et al. 2012). Here, we designed a field study to compare, by a quantitative method, the levels of mRNA expression and protein production of pollen allergens in traffic-polluted and unpolluted areas.

In the present study, we showed differences in protein and gene expression levels of *P. orientalis* allergens between pollen collected in traffic-polluted urban areas and pollen collected in unpolluted areas. Plants develop a number of defense strategies in response to environmental stressors such as pathogenic microorganisms and pests, drought, freezing temperature, ozone, and ultraviolet B (UVB) light. One of these strategies includes upregulation of certain defensive proteins, known as pathogenesis-related (PR) proteins (Armentia et al. 2002; Cortegano et al. 2004; Suárez-Cervera et al. 2008). These proteins have been classified into 17 families based on their structures and biological functions (Gorjanović 2009). About 25 % of International Union of Immunological Societies (IUIS)-recorded environmental allergens are PR proteins (Hoffmann-Sommergruber 2000; Aina et al. 2010). Evidence shows that air pollution can also act as a stressor

Fig. 3 Representative immunoblot membrane probed with specific antibodies to assess the allergenic protein levels in pollen samples from areas with heavy traffic compared to unpolluted areas collected in 2013 and 2014. *LMW* molecular weight marker; 1: Azghad; 2: Piveh Zhan; 3: Ferdoosi; 4: Vakil abad; 5: Rahnamaeei



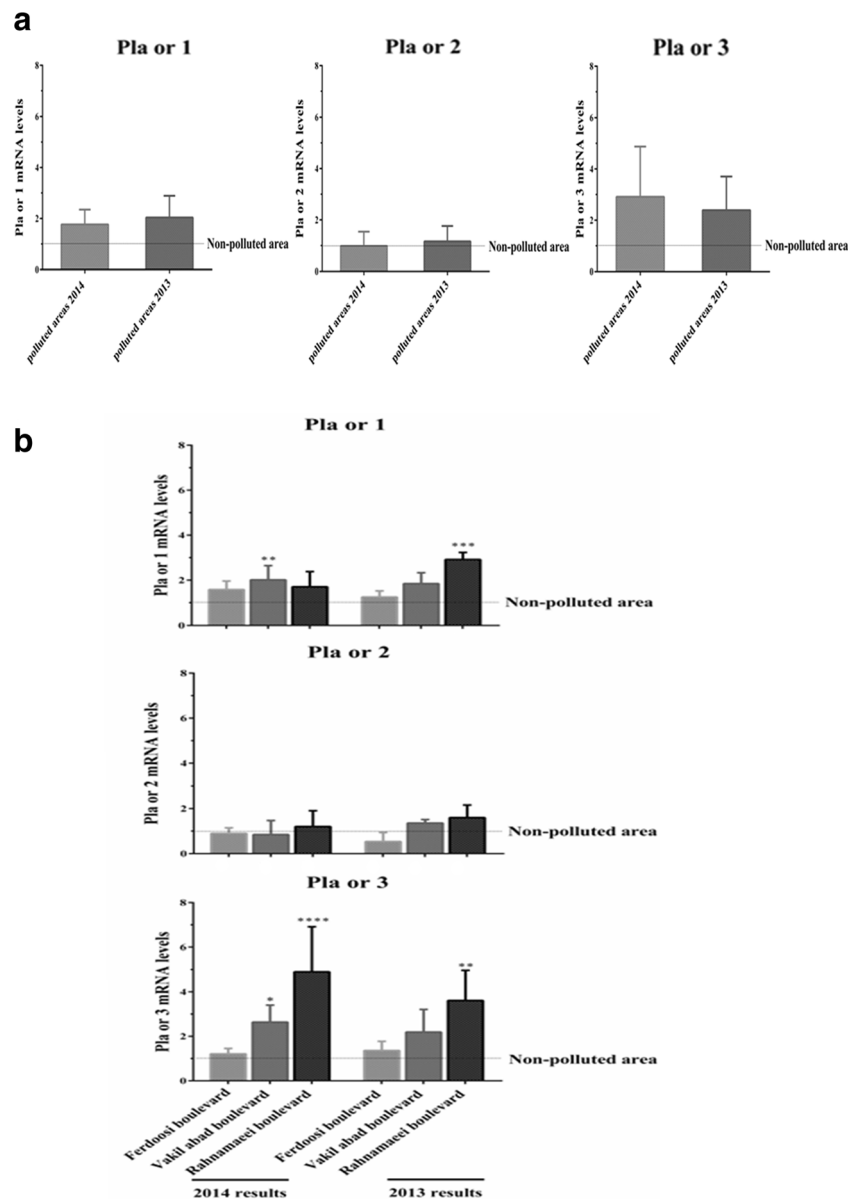
and stimulate the expression of these defensive proteins (Aina et al. 2010). In this study, we revealed greater mRNA expression of Pla or 3, as a member of the family of non-specific lipid-transfer proteins (ns-LTPs) (Fernández-Rivas 2009), in pollen collected from highly polluted sites than in unpolluted sites. ns-LTPs are 7–9 kDa soluble proteins named for their lipid-transferring function between membranes and are categorized in the PR-14 family. These findings are in agreement with the findings by Suárez-Cervera et al. (2008) and Cortegano et al. (2004), which reported higher amounts of Cup a 3 allergen in *Cupressus arizonica* pollen grown near heavy-traffic roads than in pollen from lighter-traffic roads. On the other hand, we found no significant difference in Pla or 2 mRNA expression in pollen grains from high- and low-traffic sites. Furthermore, Pla or 1 is an invertase/pectin methylesterase inhibitory protein. Some studies suggested that these proteins are involved in plant development and also in defense mechanisms (Sturm and Tang 1999; Fernández-González et al. 2010; Hong et al. 2010). The increased Pla or 1 mRNA expression levels found in polluted areas of this study might be due to their involvement in stress responses. Although Pla or 1 mRNA expression was significantly greater in pollen collected from polluted than in unpolluted areas, Pla or 3 was even greater in pollen grains from polluted areas. These results confirmed the substantial effect of traffic-

related air pollutants on Pla or 3 allergen, classified as a PR protein.

Also, our investigation demonstrated greater production of Pla or 3 protein in pollen from polluted than from unpolluted areas. These results agree with those reported by Armentia et al. (2002) and Aina et al. (2010), which showed higher amounts of allergenic proteins in pollen grown in polluted than in unpolluted conditions.

Results from field-based studies aimed at investigating the effect of air pollution on plant allergens have not been consistent. While a number of studies showed higher concentrations of allergens in pollen grains collected from unpolluted areas (Chehregani et al. 2004; Bosch-Cano et al. 2011), others found no difference between the levels of allergens in urban and rural areas (Bryce et al. 2010). This contradiction might be due to the variety of experimental conditions. For instance, previous studies have reported the results obtained from a wide range of allergens, whereas our results indicated that air pollution, as a stressor, increase the expression of Pla or 1 and Pla or 3, the allergens involved in plant defense mechanisms. On the other hand, in this study, we observed a direct relationship between the volume of traffic in the street and the detected levels of Pla or 3 mRNA sampled from that area. This observation agrees with a study by Ghiani et al. (2012) who showed far less allergenic potency of ragweed

Fig. 4 mRNA expression levels in *P. orientalis* allergens in polluted areas determined by real-time PCR. The expression level of allergens from polluted areas was normalized to those from unpolluted areas (=1). **a** mRNA expression levels of target allergens (Pla or 1, Pla or 2, and Pla or 3) in polluted areas between the years 2013 and 2014 using unpaired Student's *t* test. No significant difference was found between the results of 2 years. **b** mRNA expression levels of target allergens were compared between different areas of study (Ferdoosi, Vakil abad, Rahnamaeei) using one way ANOVA test. Pla or 3 and Pla or 1 mRNAs were significantly greater in polluted than in unpolluted areas; whereas no statically significant difference was observed in Pla or 2 mRNA expression in samples from the two areas. Results were represented as the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$



allergens from a park covered by dense vegetation than in those collected along heavy-traffic roads. In addition, Yin et al. (2011) showed that vegetation can alleviate the negative influence of air pollutants on pollen allergens by capturing gaseous pollutants, resulting in a large reduction in the levels of pollutants in vegetated areas. These reports indicate that the site of sampling can affect the study results. Another important factor is the choice of plant species. *P. orientalis* is an oriental plane tree largely cultivated along main streets of the city, a condition that favors the investigation of air pollutants on this plant. Moreover, the state under which plants have grown should be considered. For example, grasses can be easily damaged in urban areas, a negligible effect in rural districts (Armentia et al. 2002). This environmental damage might act as a stressor and stimulate the expression of PR

proteins. Finally, climatic conditions such as temperature, rainfall, and humidity are additional intrusive factors that should be the same at all study sites. We did not find any significant differences in the expression of allergens between the years 2013 and 2014. This finding is in contrast to the finding of Buters et al. (2010) who reported a 115 % difference in Bet v 1 allergen release from pollen between years 2004 and 2007. This group suggested that the difference in allergen release is mainly related to weather conditions.

Our microscopic results showed that more particles were found on the surface of pollen collected from high-traffic areas. This observation is similar to those by Chehregani et al. (2004) and Lu et al. (2014) which reported accumulation of new particles on pollen surface after exposure to air pollutants. Previous studies suggested that air pollutants can damage

the cell wall structure which might allow the release of more allergens in the environment, thus facilitating their penetration into the lower respiratory tract (Motta et al. 2006; Ghiani et al. 2012; Puc 2012). This mechanism could be a factor in the increased prevalence of allergic diseases.

Conclusion

Our study showed that air pollutants can increase the expression of two major allergens of *P. orientalis* (Pla or 1 and Pla or 3), with a considerable effect on Pla or 3, classified as a PR protein. A consequence of their release in the environment might be an increase in the prevalence of allergic diseases. These findings suggest the necessity of supporting public policies aimed at controlling traffic pollution to improve air quality and prevent the subsequent clinical outcomes and new cases of asthma. Also, avoiding outdoor activity on high pollution days can be added as part of asthmatic patient management plan.

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Compliance with ethical standards

Ethical standards All the experiments comply with the current laws of Iran.

Conflict of interest The authors declare that they have no conflict of interest.

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