Localization of Four Allergens in *Artemisia* Pollen by Immunofluorescent Antibodies

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Keywords

Pollen · Mugwort allergen · *Artemisia* · Location · Immunofluorescence

Abstract

Background: *Artemisia* pollens have a high potential to induce allergic symptoms. Seven allergen components have been identified, but only Art v 7 has been localized in the pollen grain. This study aimed to localize the allergens in the pollen grains of 4 *Artemisia* spp. **Methods:** Pollen extracts from 2 Chinese *Artemisia* spp., *A. argyi* and *A. annua*, were used to immunize BALB/c mice. Recombinant Art v 1 and Art v 3 allergens were used to select specific monoclonal antibodies (mAbs). Three mAbs were used to purify the natural allergens and were then analyzed by mass spectrometry. As reported previously, polyclonal antibodies were obtained from rabbits immunized with 3 synthesized peptides of Art an 7. Using conventional histology procedures with pollens from 4 *Artemisia* spp. (*A. argyi*, *A. annua*, *A. capilaris*, and *A. sieversiana*), allergen images were observed and recorded by fluorescence and confocal laser microscopy. **Results:** We obtained 2

specific mAbs against Art v 1, 1 against Art v 2, and 4 against Art v 3 homologs. The Art v 1 and Art v 3 homologs were mainly located on the pollen walls, and the Art v 7 homologous protein was localized intracellularly around nuclei. The location of the Art v 2 homologous protein varied across species, being intracellular around nuclei for *A. annua* and *A. argyi*, and in both the pollen wall and around nuclei for *A. capilaris* and *A. sieversiana*. **Conclusions:** Four mugwort allergens were localized in the pollen, and the major Art v 1 and Art v 3 allergens were located mainly in the pollen wall.

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Introduction

Artemisia pollens have been recognized as a major cause for late summer and autumn seasonal allergic respiratory disease worldwide, particularly in China, north of

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the Yangzi river [1]. Molecular characterization of *Artemisia* pollen allergens has revealed 7 protein molecules [2]. To date, Art v 1, Art v 2, Art v 3, and Art v 7 have been shown to be the most relevant allergens worldwide [2–8].

Pollen grains are surrounded by a complex, multilayered wall. Pollen apertures are modified regions of the pollen wall, with a significant reduction in the thickness of the outer layer (exine). Pollen allergens are highly water-soluble proteins that can be released in a humid environment, and, upon inhalation, induce an allergic reaction within minutes [9, 10]. Early immunocytochemical localization of antigens (allergens) indicated their abundance in the pollen wall of ragweed [11, 12], and in the exine and cytoplasm of birch pollen [13]. For specific allergen components, both Lol p 1 and Lol p 5 have been found in the cytoplasmic materials expelled from pollen after hydration [14]; Par j 1, an LTP of *Parietaria judaica* has been located in the cytoplasm [15], and olive pollen profilin (the Ole e 2 allergen) in the actin cytoskeleton [16]. Bet v 1, the major birch pollen allergen has been located exclusively in the cytoplasm of the pollen grain [17]. These results were found by immunolocalization with the aid of transmission electron microscopy (TEM).

For mugwort (*A. vulgaris*) pollen, only a high-molecular-weight allergen (referred to as Art v 60 kDa, known as Art v 7) [8] has been located in the mitochondria and cytoplasmic matrix by TEM immunolocalization [18]. Locations of other *Artemisia* pollen allergens have not been reported. *Artemisia* pollens from different species have been observed by light microscopy and scanning electron microscopy [19]. The pollen appears globular or oblate, a 3-lobed circular structure (diameter 16.3–36.5 µm) from the polar view and round or oblong (17.5–36.5 µm) from the equatorial view. The mugwort pollen exine has obvious double inner and outer layers, and the outer layer (3–5 µm) is thicker than the inner layer [19]. In this study, we used immunocytochemical methods with antibodies against Art v 1, Art v 2, Art v 3, and Art v 7 to localize 4 *Artemisia* pollen allergens and found 2 major allergens are present, mainly in the pollen wall.

Methods

Artemisia spp. and Protein Extract

Pollens from 5 *Artemisia* spp., *A. annua* (sweet wormwood), *A. argyi* (Chinese mugwort), *A. capilaris* (capillary wormwood), *A. gmelinii* (Russian wormwood), *A. sieversiana* (sieversian mugwort), belonging to 4 sections *Abrotanum*, *Artemisia*, *Dracunculus*, and *Absinthium* in this genus, were collected from Shanxi province in Northern China. Aqueous protein extracts of pollen

were prepared by resuspending 2 g of grains in 35 mL PBS buffer (0.14 M NaCl, 2.7 mM KCl, 7.8 mM Na₂HPO₄, 1.5 mM KH₂PO₄) and shaking for 12 h at 4 °C. Extracts were centrifuged at 10,000 g for 10 min at 4 °C, filtered through 0.22-µm filters (Millipore), and the concentration of protein extracts was then determined using the BCA method.

Production and Purification of Allergen Component-Specific Antibodies

Five BALB/c mice (experimental animal use permission, SCXK (HU) 2012-002, China) were immunized with 100 µg of pollen extracts of *A. argyi* and *A. annua* in incomplete Freund's adjuvant, followed by 6 subcutaneous boosters with 50 µg of pollen extracts at intervals of 7–14 days. Antibody-producing hybridoma cells were screened by ELISA and later by Western blot with immunized pollen extracts, recombinant (r)Art v 1.0101 [2, 20] and Art v 3 allergen rArt v 3.0201 [21], to select specific monoclonal antibodies (mAbs). mAbs were purified by protein G column and isotype determination as described previously [22]. One of the specific mAbs for Art an 1, Art ar 2, and Art an 3 was used to purify the target protein on a CNBr-activated Sepharose™ 4 fast flow column (71–5000–15AF preactivated media, spin column, 10 mL, C006727, GE Healthcare, USA). The purified protein was first tested with *A. argyi*-allergic sera pool by ELISA as described previously [22] to confirm it is an allergen, and then analyzed by LC-MS (Applied Biosystem MALDI-TOF/TOF 5800) or LC-MS/MS (Thermo Scientific Q Exactive) for its identity-matching to known *Artemisia* allergens.

Production of polyclonal antibodies has been reported recently [8]. Three synthesized peptides (T1: aa1–14, APDITDGGDKP-GPE; T2: aa508–520, LYPPFTTHGFSQN; T3: aa 377–389, KGTS-GWEDATEANL, which were predicted on the surface of the protein with high antigenic potential by DNASTar-Lasergene-Protean program) of Art an 7 were used to immunize 2 New Zealand rabbits, injecting 500 µg in incomplete Freund's adjuvant, followed by 3 subcutaneous boosters of 250 µg at intervals of 7–14 days. The quality was checked using both Western blot and ELISA with purified natural Art an 7 allergen. The polyclonal antibodies were purified by chromatography using the sensitized peptide through a Sulfo-Link-gel column. The antibodies were produced by Hua An Biotech Ltd., Hangzhou, China.

Immunoblot and ELISA Binding Experiments

These methods follow a previously published protocol [22]. Crude pollen extracts were analyzed by Tricine-SDS-PAGE and visualized by staining with Coomassie brilliant blue R-250. For IgG immunoblot analysis, proteins were separated on 12% acrylamide gels and electroblotted onto 0.2-µm PVDF membranes (Bio-Rad, USA). Pollen extracts from 5 species were used to analyze binding to mAbs by ELISA. ELISA plates (Corning, USA) were coated with 100 µL of 5 µg/mL pollen extracts or 1 µg/mL recombinant and purified allergen in PBS buffer (pH 8.3). After blocking with 100 µL 5% skimmed milk, 100 µL serum pool was added and a negative serum pool used as a control. The plates were washed with PBS buffer containing 0.5% Tween-20, and then goat anti-mouse or anti-rabbit IgG coupled with HRP (1:3,000 in PBS buffer) was added and bound IgG was detected using TMB (3, 3', 5, 5'-tetramethylbenzidine). ELISA optical density (OD) readings were measured using the colorimetric reaction at 450 nm (Multiskan FC, Thermo Fisher).

Fig. 1. Immunoblot analysis of antibodies derived from mice immunized with pollen extracts from Chinese mugwort and sweet wormwood. **a** ArE, *A. argyi* pollen extract. Lanes 1–5 immunized mice. **b** AnE, *A. annua* pollen extract. Lanes 6–10 immunized mice. **c** Three mAbs derived from mouse No. 3 immunized with ArE. **d** Three mAbs were derived from mouse No. 6 and 1 from mouse No. 8.

Immunolocalization of Allergens on Pollen

Four *Artemisia* spp. pollen grains were firstly fixed in FAA for 24 h at 4 °C and centrifuged to collect the pollen and melted 2% agarose gel was added. All subsequent steps were performed at room temperature if not stated specifically. The gel block with dispersed pollen was dehydrated with alcohol series and dimethylbenzene, and then embedded at 65 °C in paraffin wax. Sample sections of 5 µm were washed in dimethylbenzene and rehydrated in an alcohol series. The rehydrated samples were incubated for 5 min in autofluorescence quencher (0.3% Sudan black B) after infiltrating with antigen retrieval solution (EDTA, pH = 8, G1206, Wuhan Servicebio Tech, China).

Sections of 5 µm were blocked for 30 min at room temperature in 3% BSA buffer, followed by overnight incubation at 4 °C with selected mAbs (diluted 1:200 in PBS buffer) against the major mugwort allergens, and were then washed in PBS buffer 3 times. For immunofluorescence labeling, sections were incubated for 50 min in the dark at room temperature with Cy3 conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Goodbio Technology, China) diluted 1:300 in PBS, and then stained 10 min at room temperature in the dark with DAPI solution and washed. Finally, sections were mounted with antifade mounting medium (Beyotime, China) and observed with a Nikon ECLIPSE C1 confocal laser scanning microscope, with a HeNe (543 nm) laser for Cy3 excitation and a blue diode (405 nm) for DAPI excitation, by using a 40×/0.95 NA objective. Image processing was performed with a Nikon DS-U3 system. Single optical sections were observed. Pollen of *A. annua* without antibody or antifade treatment was observed for autofluorescence. *Chenopodium alba* pollen, mAb A7-1 specific to peach allergen Pru p 3 [22], and α-SMA mAb to human actin were used as negative controls.

Results

Immunization of Mice with Mugwort Pollen Extracts

Both Western blot and ELISA were used to check the immunization with pollen extract of 2 Chinese *Artemisia* spp. (*A. argyi* and *A. annua*). Four protein bands, around 10, 16, 22, and 40 kDa were recognized by IgG from the sera of immunized mice, with the 10- and 22-kDa bands being more common (Fig. 1a, b). The preselective threshold of the polyclonal antibody ELISA OD 450-nm values with pollen extracts and recombinant allergens were >1.0 and >0.35, respectively (Table 1). Mice Nos. 1, 2, 5, 7, 9, and 10 were discarded as they reacted weakly or to fewer bands on Western blot. Mice Nos. 3, 4, 6, and 8 were initially selected for a further monoclonal selection process, but No. 4 was dropped because it did not pass the ELISA selection procedure.

Selection of mAbs

Screening by ELISA of mugwort rArt v 1 gave 2, and Art v 3 showed 4 mAbs (Tables 1, 2), confirmed by Western blot (Fig. 1c, d). With the *A. argyi* extract, 3 mAbs were obtained, 2 (A9-G10 and A11-B2) against 10 kDa responding to Art v 3, and C9-C1 against a 16- to 17-kDa which was highly identical to Art v 2 and used to purify it by immunoaffinity from *A. argyi* (online sup-

Table 1. ELISA screening of serum from immunized mice with 2 *Artemisia* pollen extracts and 2 allergen components (OD at 450 nm)

Dilution	Mouse #3			Mouse #6			Mouse #8		
	<i>A. argyi</i> extract	rArt v 1	rArt v 3	<i>A. annua</i> extract	rArt v 1	rArt v 3	<i>A. annua</i> extract	rArt v 1	rArt v 3
1:200	1.48	1.01	1.83	1.87	1.54	1.87	1.77	1.48	1.80
1:1,000	1.28	0.36	1.41	1.79	0.80	1.82	1.52	0.72	1.52
1:5,000	0.87	0.11	0.91	1.49	0.19	1.56	0.76	0.15	0.88
1:10,000	0.60	0.07	0.67	1.16	0.09	1.05	0.54	0.09	0.46
1:20,000	0.42	0.05	0.43	0.84	0.05	0.83	0.26	0.05	0.27

Coated with 5 µg/mL pollen protein extracts or 1 µg/mL recombinant (r) allergen.

Table 2. Subcloned cell lines selected with pollen extracts and two recombinant allergens by ELISA

Antigen source	Subclone	OD450		
		extract	rArt v 1	rArt v 3
<i>A. argyi</i>	A9	1.41	0.04	1.67
	A11	1.25	0.03	1.80
	C9	0.61	0.04	0.04
<i>A. annua</i>	5	1.86	0.05	1.87
	A1	1.43	0.24	2.24
	A7	1.31	1.35	0.05
	A2	1.35	0.05	1.82

Coated with 5 µg/mL pollen protein extracts or 1 µg/mL recombinant (r) allergen.

pl. Fig. S1; for all online suppl. material, see www.karger.com/doi/10.1159/000497321). The purified protein was reactive to mugwort-allergic patients' serum. It has been shown to be an Art v 2 homologous allergen by mass spectra and designated Art ar 2 by the WHO/IUIS Nomenclature Subcommittee (<http://allergen.org/viewallergen.php?aid=907>). Sera from mice immunized with *A. annua* were screened using rArt v 1 and the mAbs A7-G4-E6 and A1-D10 were obtained. mAbs 5-E10 and A2-B8 were selected using rArt v 3. A7-G4-E6 and A2-B8 mAbs (Art an 1 and Art an 3) derived from *A. annua* pollen were used to purify the expected proteins by affinity chromatography, and the obtained 2 proteins matched Art an 1 and Art an 3 by LC-MS/MS (online suppl. Fig. S2). In total, we obtained 2 mAbs against Art v 1 homologs, 1 to Art v 2 homologs, and 4 to Art v 3 homologs. All mAbs were IgG1 isotype. Art v 1 mAbs have high titers to their target allergen component, me-

dium titers to pollen extracts of 4 species, and significantly low titers to *A. gmelinii* on ELISA (Fig. 2). One Art v 2 mAb was negative to Art v 1 and Art v 3, had medium titers to 3 species, and very low titers to another 2 species. Four Art v 3 mAbs had a different binding pattern among 5 different *Artemisia* spp. pollen extracts, and A11-B2 reacted only to rArt v 3 and very weakly to pollen extracts (Fig. 2).

Selection of Antibodies Suitable for Immunolocalization

Autofluorescence of *A. annua* pollen was observed in red, green, and blue (online suppl. Fig. S3), so the autofluorescence quencher (Sudan Black B) was used before labeling with the antibodies. Screening with the 7 mAbs, mAb A7-G4-E6 was suitable to localize Art v 1, C9-C1 for Art v 2, and mAb A9-G10 to localize Art v 3 homologous proteins. Mugwort mAbs A1-D10 (to allergen Art an 1) and 5-E10 (to allergen Art an 3) gave no signal with *A. annua* pollen, and autofluorescence was also absent after using the quencher Sudan Black B (online suppl. Fig. S4). Other mAbs also failed to recognize proteins in *A. annua* pollen (online suppl. Fig. S4). Negative controls with weed pollen *C. alba* of 2 selected mAbs gave very weak signals (online suppl. Fig. S5).

While 6 *Artemisia* spp. were initially used in the immunolocalization experiment *A. gmelinii* and *A. vulgaris* gave poor images, probably due to the poor quality of the pollen grains. So here we showed the results from 4 species. Mugwort defensin-like protein (Art v 1) was abundant in the pollen wall (with a stronger signal on the outside of pollen wall), more on the ridge than in the furrow, but was low in the 3 germination pores and cytoplasm. There was no visible difference in the 4 species, apart from pollen size (Fig. 3; online suppl. Fig. SS6–S8). Art v

Fig. 2. Specificity test of 2 Art v 1 mAbs, 1 Art v 2 mAb, and 4 Art v 3 mAbs (on the horizontal axis) in 5 different *Artemisia* pollen extracts and recombinant (r) Art v 1 and Art v 3 allergens (3 replicates of the extracts, error bars refer to SD). There was no replicate for rArt v 1 and rArt v 3 ELISA.

2 homologs were present mainly in the cytoplasm surrounding the nuclei in the *A. argyi* and *A. annua* pollens (Fig. 3; online suppl. Fig. S6), and in both the pollen wall and around nuclei in *A. capillaris* and *A. sieversiana* (online suppl. Fig. S7, S8). The mugwort lipid transfer protein (Art v 3) was also located mainly in the pollen wall, with some in the cytoplasm (Fig. 3; online suppl. Fig. S6, S8), while for the pollen of *A. capillaris*, there was a strong signal in both the wall and cytoplasm (online suppl. Fig. S7). Art an 7 homologous allergens were found mainly in the cytoplasm around nuclei (Fig. 3; online suppl. Fig. S6–S8).

Discussion

In this study, we localized 4 mugwort allergens in pollen, each with different patterns of location. Art v 1, a defensin-like protein, gave very clear and strong signals in the pollen wall, estimated as 2- to 3- μ m thick, which is close to the average thickness of exine (range 3.1–5.2 μ m) [19]. In the same Compositae family of mugwort, *Ambrosia artemisifolia* also has a clear pollen wall [23]. In plants, the defensin-like pollen protein was thought to be a major protein generally located in the pollen coat [24]. As Art v 3, a nonspecific lipid transfer protein, is located mainly in the pollen wall, these 2 ma-

for allergens are likely to be important protein constituents to protect the pollen and rapid leaching once pollens are drawn into the respiratory system of patients. Art v 2 homologs are less abundant in mugwort pollens, and their location varied across different *Artemisia* spp., some with stronger signals near the nuclei. Art v 7 is a putative galactose oxidase of 62 kDa, referred to as a Art v 60-kDa glycoprotein [8], and previously as Art v I [25], and it has been located in the mitochondria and cytoplasmic matrix by electron microscopy [18]. Our finding of *Artemisia* pollen group 7 allergens (Art an 7 and Art v 7) in the cytoplasm is consistent with previous reports [18]. The presence of Art v 1 and Art v 3 in the cytoplasm might indicate their initial synthesis there, and they are then transported to the pollen wall during pollen maturation.

Due to the quick release of pollen allergens in a humid environment, fixation in aqueous solution affects the allergen distribution, so anhydrous preparation has been recommended [9]. We followed the conventional fixing procedure, which resulted in some pollen rupture as some nuclei were seen outside the pollen wall. Plant pollen walls harbor some allergens, as reviewed recently [26], and our research can add defensin-like protein to the list.

We successfully obtained mAbs with pollen-allergic protein extract and subsequent selection with allergens of

Fig. 3. Confocal laser scanning microscopy showing the immunofluorescence (Cy3, red) of 4 allergens in pollen grains of *A. argyi* by labeling with the specific antibodies. **a** Defensin-like protein Art ar 1 by mAb A7-G4-E6. **b** Pathogenesis-related protein Art ar 2 by mAb C9-C1. **c** Nonspecific lipid transfer protein Art ar 3 by mAb A9-G10. **d** Putative galactose oxidase allergens Art ar 7 by specific pAb (T1). Nuclei are in blue. $\times 400$. Excitation (543 nm) and emission (590 nm) laser for Cy3. Laser excitation 405 nm and emission (420 nm) for DAPI. W, pollen wall; N, pollen nuclei (in blue); A, pollen aperture.

known identity. The mice immune system reaction to mugwort pollen extract demonstrated that allergens to have greater immunogenicity than nonallergenic proteins. In addition, mAbs selected with recombinant allergen reacted as expected to recombinant proteins and natural allergens. The immunogenicity of Art v 3 was the highest (Table 1). Our approach for producing mAbs with pollen extract immunization can overcome the shortage of purified natural and recombinant allergen. It was also an advantage to be able to use the obtained mAb

to purify potential unidentified allergens by immunoaffinity methods, and then determine the nature of the protein by mass spectrometry and peptide-matching with the aid of the transcriptome [8].

The mAbs from this research could be used for other immunolocalization, immunoaffinity purification of natural allergens, and quantity measurements, and perhaps pollen count in flow cytometry [27]. Pollen data are an important tool for the prevention of allergic symptoms [28]. In nature, the quality and quantity of a certain aller-

gen is variable and dynamic: indirect molecular composition changes have been observed in *A. vulgaris* pollen under traffic pollution [29]. In the new era of molecular aerobiology [30], new tools are required to monitor true allergenic pollen and assess pollen allergic potency in different geographic regions as well as the impact of climate and plant-related factors.

Conclusion

We obtained mAbs against Art v 1, Art v 2, and Art v 3 homologous allergens. The most important allergens, Art v 1 and Art v 3, were mainly located on the pollen wall, and Art v 2 was present in the cytoplasm or in both the pollen coat and cytoplasm, depending on the species. We confirmed the localization of Art v 7 homologous allergen intracellularly around nuclei.

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Statement of Ethics

Experimental BALB/c mice animal use was approved by permission SCXK (HU) 2012–002, China.

Disclosure Statement

All authors declare that they have no relevant conflicts of interest.

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Author Contributions

ZSG, RVR and FF conceived and designed the study. LZ, LG and BYG collected pollens, performed natural protein purification. WYF, LZ and SDW did pollen transcriptome/proteome analysis and gene cloning and sequencing. GG, FF and SAV performed the recombinant allergen production. ZSG, WYF, JZ performed the antibody selection, specificity test and immunochemical localization. ZSG, WYF, GG and RvR drafted the manuscript in close collaboration with all co-authors.

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