

Role of the polypeptide backbone and post-translational modifications in cross-reactivity of Art v 1, the major mugwort pollen allergen

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Abstract

Artemisia vulgaris (mugwort) is one of the main causes of late summer pollinosis in Europe, with >95% of patients sensitized to the glycoallergen Art v 1. Despite the importance of this allergen, little is known about its cross-reactive behavior. Here we investigated the occurrence of conserved Art v 1 antigenic determinants in sources known to display clinically relevant cross-reactivity with mugwort pollen. For this purpose, monoclonal antibodies specific for a cysteine-stabilized epitope of the Art v 1 defensin domain and for carbohydrates attached to the proline domain were produced by hybridoma and phage display technologies. Using polyclonal Art v 1-specific rabbit sera and antibodies against both the Art v 1 carbohydrate and polypeptide moieties, we could identify cross-reactive structures in pollen from botanically related Asteraceae weeds (*Artemisia absinthium*, *Helianthus annuus* and *Ambrosia* sp.). Homologous allergens were also recognized by IgE from mugwort-sensitized patients and the reactivity could be decreased by serum pre-incubation with natural and recombinant Art v 1. As no cross-reactive structures could be found in foods associated with mugwort pollinosis, we conclude that Art v 1 is poorly involved in mugwort cross-reactivity to food allergens.

Keywords: ETH-2 phage library; glycoallergen; monoclonal antibody; mugwort pollen allergy; pDAP2/S vector; phage display.

Introduction

Among pollinosis patients the prevalence of sensitization by mugwort (*Artemisia vulgaris*) ranges from 10% to 14% (Wopfner et al., 2005). In addition, several studies have reported on cross-reactivity with proteins originating from pollen (Fernandez et al., 1993; Hirschwehr et al., 1998; Wopfner et al., 2005), latex (Fuchs et al., 1997), and various plant-derived food allergen sources (Egger et al., 2006). So far, several mugwort pollen allergens have been characterized to varying degrees (Wopfner et al., 2005). Among these, mugwort LTP, profilin, and polcalcin have been convincingly demonstrated to be involved in IgE cross-reactivity (Lombardero et al., 2004; Egger et al., 2006; Gadermaier et al., 2008; Wopfner et al., 2008). However, more than 95% of mugwort-sensitized patients react to the major allergen Art v 1 comprising an amino-terminal globular domain displaying high homology to plant defensins, and a carboxy-terminal domain rich in hydroxyproline residues with an extended left-helical shape resembling that of collagen. Heavy O-glycosylation of the proline-domain accounts for approximately 30–40% of the molecule's mass and has been demonstrated to be important for IgE recognition (Himly et al., 2003). Recently, a detailed characterization of Art v 1 O-glycans has been reported (Leonard et al., 2005), revealing two patterns of glycosylation on the proline-domain: (i) single β -arabinofuranoses linked to hydroxyproline residues; and (ii) a large hydroxyproline-linked arabinogalactan composed of a short β 1,6-galactan core substituted by a variable number of α -arabinofuranose residues, tentatively designated type III arabinogalactan (Himly et al., 2003; Leonard et al., 2005).

Despite its importance as an allergen, studies addressing the IgE cross-reactivity of Art v 1 are still lacking. Here we investigated the occurrence of Art v 1 antigenic determinants in allergenic sources known to display cross-reactivity with mugwort. For this purpose, a monoclonal antibody (mAb) raised against the Art v 1 polypeptide and Art v 1 carbohydrate-specific antibodies were produced by hybridoma and phage display technologies.

Results

Hybridoma production and characterization of a mAb specific for the Art v 1 defensin domain

Using genetic immunization and conventional hybridoma fusion techniques, we were able to produce a murine mAb directed against the Art v 1 polypeptide. ELISA-based sub-typing revealed this antibody to be of the IgM subclass. For use in immunoassays, optimal working

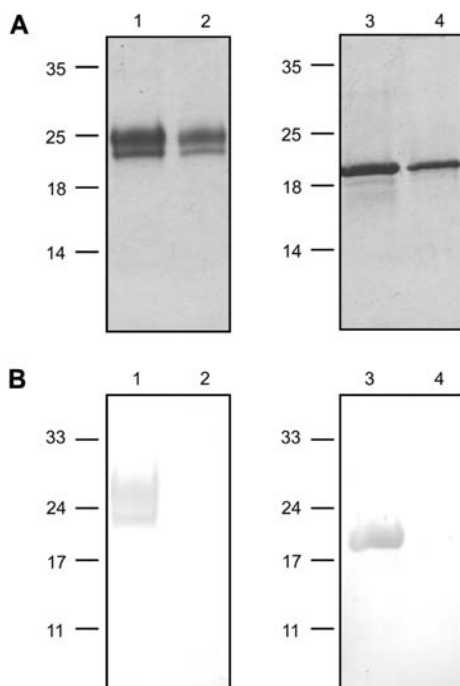


Figure 1 Specificity of anti-defensin mAb produced by genetic immunization. Coomassie Blue staining (A) and immunoblot analysis using anti-defensin mAb (B) for nArt v 1 (1), reduced and alkylated nArt v 1 (2), rArt v 1 (3), and reduced and alkylated rArt v 1 (4).

concentrations were determined for ELISA (1:5) and immunoblot analysis (1:50) using mugwort pollen extract, as well as natural (nArt v 1) and recombinant (rArt v 1) Art v 1. As shown in Figure 1, the mAb recognized both natural and recombinant Art v 1. These results could be verified under native ELISA conditions (data not shown). Antibody binding to both natural and recombinant Art v 1 could be abolished after allergen reduction and alkylation, suggesting specificity for a cysteine-stabilized epitope of the defensin domain. Therefore, this antibody was designated anti-defensin mAb.

Phage display production and characterization of a mAb specific for Art v 1 α -arabinoses

Subtractive phage display was used to isolate five antibodies in single-chain fragment-variable (scFv) format specific for Art v 1 glycans from the synthetic human recombinant antibody library (ETH-2) (Viti et al., 2000; Silacci et al., 2005). Sequence evaluation revealed that the five clones consisted of the DP47 germline gene seg-

ment for the heavy chain and the DPL16 germline gene segment for the light chain, joined by a flexible peptide linker. According to differences in the complementary determining region 3 (CDR3) of heavy and light chains, clones could be subdivided into group 1 (1, 4 and 5) and group 2 (2 and 3) clones (Figure 2). Respective fragments were sub-cloned into pDAP2/S and expressed in *Escherichia coli* TG1, resulting in 75-kDa scFv antibodies (Figure 3A) harboring carboxy-terminal hexahistidine- and alkaline phosphatase (AP)-tags, thus allowing single-step purification by immobilized metal chelate affinity chromatography (IMAC) and direct signal detection when used in immunoassays. Amounts of purified scFv-AP fragments ranged from 0.5 to 4 mg/l culture. Optimal working dilutions were determined for immunoblot (10 μ g/ml) and ELISA (2.5 μ g/ml). Immunoblots using purified nArt v 1 and mugwort pollen extract showed a band at approximately 28 kDa (Figure 3B) and, as expected, no signals were obtained with rArt v 1. These results were confirmed under native conditions in ELISA experiments (data not shown). Furthermore, the epitope specificity of scFv-AP antibodies was investigated using de-arabinosylated and de-glycosylated Art v 1 (Leonard et al., 2005) in immunoblot experiments (Figure 3C). ScFv-AP antibodies failed to recognize both de-arabinosylated and de-glycosylated Art v 1 preparations, indicating specificity for Art v 1 α -arabinoses.

Art v 1 cross-reactive structures can be found in botanically related Asteraceae weeds

The antibodies produced and polyclonal Art v 1-specific rabbit sera (Himly et al., 2003) were subjected to immunoblot analyses to screen for conserved Art v 1 antigenic determinants in pollen extracts from Asteraceae weeds and birch, and extracts from mugwort pollinosis-associated food allergens (Egger et al., 2006). As shown in Table 1, cross-reactive structures were exclusively identified in the pollen of plants belonging to the Asteraceae family, i.e., wormwood (*Artemisia absinthium*), sunflower (*Helianthus annuus*), and different species of ragweed (*Ambrosia* sp.). In more detail, Art v 1 defensin and α -arabinose-specific mAbs bound cross-reactive polypeptide and glycan compounds migrating at an apparent molecular weight of 28 kDa for both wormwood (Figure 4A) and sunflower (Figure 4B) pollen. The former presumably represents an Art v 1 homolog in *Artemisia absinthium*, and the latter the previously described sunflower SF18 protein (Domon et al., 1990). As expected, polyclonal Art v 1-specific rabbit sera recognized similar struc-

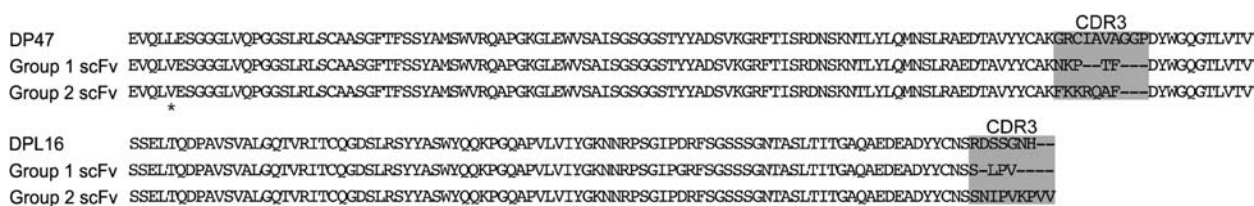


Figure 2 Sequence evaluation of scFv. Phage display antibodies consist of DP47 and DPL19 germline gene segments for heavy and light chains, respectively. Owing to differences in the CDR3 (gray box), antibodies can be subdivided into two groups. Asterisks indicate amino acid exchanges from L to V in the heavy chain and from D to G in the light chain.

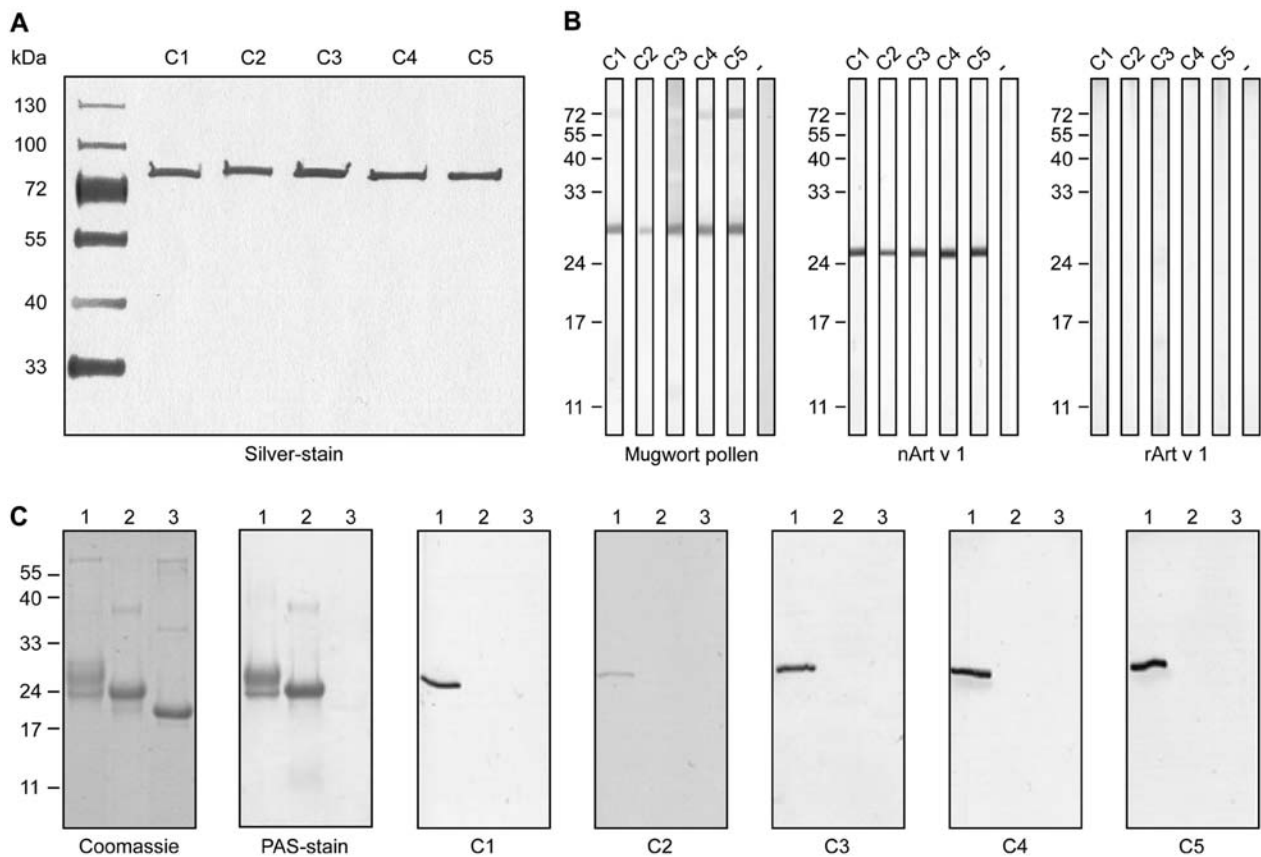


Figure 3 Purity, reactivity, and specificity of scFv-AP antibodies.

Five clones (C1–C5) were produced as AP-tagged antibodies and purity was assessed by SDS-PAGE and silver staining (A). Reactivity (B) and specificity (C) using nArt v 1 (1), de-arabinosylated Art v 1 (2), and de-glycosylated Art v 1 (3) were tested by immunoblot analysis. The presence of a carbohydrate moiety on Art v 1 molecules was assessed by staining with periodic acid Schiff's reagent (PAS).

tures. In addition, polyclonal rabbit antibodies weakly bound to proteins of 24–30 kDa in pollen extracts from different *Ambrosia* species (Table 1).

As illustrated in Figure 4, IgE antibodies from mugwort-sensitized patients also bound Art v 1 cross-reactive components from wormwood and sunflower. Moreover,

Table 1 Identification of Art v 1 conserved antigenic determinants in allergen sources known to display cross-reactivity with mugwort.

Allergen source	Botanical family	Allergen source		Anti-defensin mAb	scFv-AP	Rabbit serum
Pollen	Asteraceae	<i>Artemisia vulgaris</i>	Mugwort	+	+	+
		<i>Artemisia absinthium</i>	Wormwood	+	+	+
		<i>Ambrosia artemisiifolia</i>	Common ragweed	-	-	+
		<i>Ambrosia psilostachya</i>	Cuman ragweed	-	-	+
		<i>Ambrosia trifida</i>	Giant ragweed	-	-	+
		<i>Helianthus annuus</i>	Sunflower	+	+	+
		Fagales	<i>Betula verrucosa</i>	Birch	-	-
	Food	Asteraceae	<i>Helianthus annuus</i>	Sunflower	-	-
Apiaceae		<i>Apium graveolens</i>	Celery	-	-	-
		<i>Daucus carota</i>	Carrot	-	-	-
		<i>Carum carvi</i>	Caraway	-	-	-
		<i>Foeniculum vulgare</i>	Fennel	-	-	-
		<i>Coriandrum sativum</i>	Coriander	-	-	-
Piperaceae		<i>Piper nigrum</i>	Pepper	-	-	-
Solanaceae		<i>Capsicum annuum</i>	Bell pepper	-	-	-
Anacardiaceae		<i>Mangifera indica</i>	Mango	-	-	-
Liliaceae		<i>Allium cepa</i>	Onion	-	-	-

Immunoblotting using mAb anti-defensin, scFv-AP specific for Art v 1 α -arabinoses, and Art v 1-specific rabbit sera revealed Art v 1 cross-reactive structures (+) to occur exclusively in the pollen of Asteraceae weeds. No signals (-) were obtained for birch pollen and mugwort pollinosis-associated food allergens.

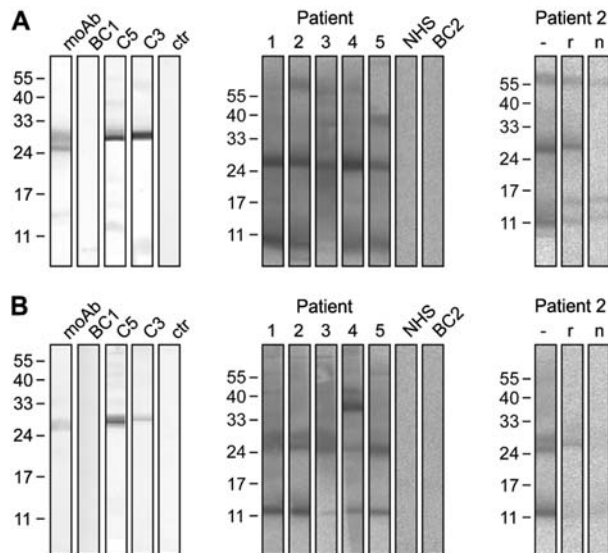


Figure 4 Identification of Art v 1 cross-reactive structures in botanically related weed pollen.

Immunoblot analysis revealed Art v 1 cross-reactive structures in pollen of *Artemisia absinthium* (A) and *Helianthus annuus* (B) using an anti-defensin mAb as well as group 1 (clone C5) and 2 (clone C3) scFv-AP antibodies (left chart). Similar signals were obtained in IgE immunoblots using sera ($n=5$) of mugwort-sensitized patients (middle chart). Binding of patients' IgE could be reduced by serum pre-incubation with recombinant (r) or natural (n) Art v 1 (right chart). Controls with normal human serum (NHS), no antibodies (ctr), or secondary antibodies only (BC1 and 2) were performed.

IgE reactivity could be reduced by pre-incubation of patient sera with 20 μ g of natural or recombinant Art v 1. Interestingly, in some cases natural Art v 1 showed higher potency in decreasing IgE reactivity (Figure 4), which strongly suggests that carbohydrate-specific antibodies are involved in epitope recognition. Signals obtained in the low-molecular-weight range, which could be partially inhibited to the same degree by natural and recombinant Art v 1, are likely to represent degradation of the intact cross-reactive molecules. Indeed, we frequently observed such truncations of mugwort pollen Art v 1 (unpublished observations). Notably, no Art v 1 cross-reactive structures could be detected in birch pollen or foods associated with celery-mugwort-spice syndrome, indicating poor involvement of Art v 1 in mugwort cross-reactivity.

Discussion

As mentioned above, more than 95% of mugwort-sensitized patients react to the major allergen Art v 1 (Himly et al., 2003). In accordance with our results, both the Art v 1 polypeptide, especially the molecule's defensin domain (Dedic et al., 2009), and carbohydrate antigenic determinants have been shown to be involved in patient IgE recognition (Himly et al., 2003). In particular, Art v 1 mono- β -arabinosylated hydroxyprolines have been suggested to bear cross-reactive carbohydrate determinants (Leonard et al., 2005; Wopfner et al., 2005). However, studies on carbohydrate-specific antibodies have mainly focused on N-glycans. The first allergenic protein studied

in more detail for its allergenic N-linked carbohydrates was bee venom phospholipase A2 (Weber et al., 1987). It is now well established that xylose and fucose residues are crucial elements for both IgE recognition and cross-reactivity (Tretter et al., 1993; van Ree and Aalberse, 1993). More recently, celery Api g 5 has been reported to be capable of binding human IgE and activating basophils exclusively via its N-glycans (Bublin et al., 2003). In general, approximately 20% of allergic patients display N-glycan-specific IgE, but despite the wide distribution of N-glycosylated proteins in allergenic pollens, foods, and insect venoms, these antibodies do not appear to cause clinical symptoms in most, if not all patients. Thus, the overall impression is that carbohydrate-specific IgE antibodies are of poor biological activity, and their clinical relevance is doubted (Mari et al., 1999; Malandain, 2005; Altmann, 2007). Nonetheless, although occurring less frequently, this might be different for O-glycans such as those found on Art v 1. In fact, this theory is supported by previous *in vitro* and *in vivo* studies that investigated the impact of Art v 1 O-glycans on allergic patients. Although inducing comparable activation of allergen-specific T cells, *in vitro* IgE-binding is slightly higher for nArt v 1 than for its recombinant counterpart lacking the natural molecule's carbohydrate moiety (Jahn-Schmid et al., 2002; Schmid-Grendelmeier et al., 2003). Moreover, Schmid-Grendelmeier et al. (2003) demonstrated that rArt v 1 elicited reduced responses in skin and nasal provocation tests in 32 mugwort-sensitized patients. In fact, 18.75% of the individuals tested exhibited drastically stronger responses in skin prick tests to natural than to recombinant Art v 1, indicating that O-linked carbohydrates might contribute to the development of clinical symptoms for several mugwort-allergic patients. However, detailed studies on the clinical significance of Art v 1 O-glycans and the impact of α - and β -arabinoses are still lacking. Moreover, although allergy to mugwort has been associated with adverse reactions upon contact with many other plant-derived pollen and foods, little is known about the underlying cross-reactive molecules. To investigate the potential involvement of Art v 1 and the role of both its polypeptide backbone and carbohydrates, we produced two different mAbs. (i) Conventional hybridoma fusion was used to generate a mAb with specificity for a cysteine stabilized epitope of the Art v 1 defensin domain. (ii) Using phage display technology, and therefore bypassing animal immunization, we isolated five Art v 1 carbohydrate-specific scFv antibodies. Notably, O-linked carbohydrate-specific mAbs have been extensively used to characterize type II arabinogalactans from plant proteoglycans (Anderson et al., 1984; Yates et al., 1996; Speranza et al., 2009). To the best of our knowledge, the scFv isolated in the present study represents the first antibody displaying specificity for α -arabinoses on type III arabinogalactans as found on Art v 1. AP conjugation allowed convenient direct detection of scFv. Using these tools, immunoblot analysis revealed Art v 1 cross-reactive polypeptide and carbohydrate structures in pollen from closely related Asteraceae (wormwood and sunflower), but not in mugwort-associated food allergen sources. These structures were also recognized by polyclonal Art v 1-specific rabbit sera (Himly et al., 2003). In

addition, rabbit sera bound to components in pollen extracts from several species of *Ambrosia*, a genus that also belongs to the Asteraceae family. However, as *Ambrosia* molecules were not recognized by the mAbs used in the present study, they seem to differ from Art v 1 in both their glycosylation pattern and the defensin domain epitope recognized by our mAb. In fact, it has been reported that cross-reactivity between Art v 1 and its homolog in *Ambrosia artemisiifolia* pollen is rather limited (Asero et al., 2006). Taken together, our data suggest that Art v 1 is not extensively involved in mugwort cross-reactivity. Nevertheless, the tools described for this study could help to clarify the specific IgE recognition and cross-recognition of glycoallergens, and thus have direct implications for the development of new applications in allergy diagnosis and therapy.

Materials and methods

Patients and sera

Mugwort pollen-allergic patients from Vienna (Austria) were selected on the basis of typical case history, positive *in vivo* skin prick test, *in vitro* IgE detection (RAST class >3), and immuno-reactivity to Art v 1.

Allergens and extracts

Aqueous pollen extracts and purified proteins were obtained and characterized as described by Himly et al. (2003) and Leonard et al. (2005). Food extracts were prepared by pulverization at -192°C and homogenization in 20 mM potassium phosphate, pH 7, 13.3 mM diethyldithiocarbamic acid, 2% (w/v) polyvinylpyrrolidone, and Complete Protease Inhibitor (Roche, Basel, Switzerland).

Production of anti-defensin mAb

For anti-defensin mAb production, BALB/c mice (Forschungsinstitut für Versuchstierzucht, Himberg, Austria) were immunized with a gene vaccine encoding synthetic codon-optimized Art v 1 (pCMV – humArt; gb: AF502559) (Bauer et al., 2003). Aliquots of 2 µg of vaccine were administered intraperitoneally three times at 2-week intervals using a Helios gene gun (Bio-Rad, Hercules, CA, USA). At 4 days and 1 day prior to sacrifice, mice were boosted with 5 µg of natural Art v 1 (nArt v 1) adsorbed on Alu-Gel-S Suspension (Serva, Heidelberg, Germany). All animal experiments were conducted according to National Guidelines approved by the Austrian Ministry of Science (BMBWK-66.012/0015-BrGT/2005). Hybridoma fusion was conducted according to standard protocols (Kohler and Milstein, 1975) using splenic lymphocytes and the BALB/c myeloma cell line P3X63Ag8.653 (ATCC, Manassas, CA, USA).

Antibody isolation by phage display

An aliquot of 10¹³ phages of the synthetic human recombinant antibodies library (ETH-2) (Viti et al., 2000; Silacci et al., 2005) was pre-incubated for 2 h with 100 µg/ml rArt v 1 in phosphate-buffered saline (PBS). Subsequently, phages were used for panning, performed by 2-h incubation in immunotubes (Nunc, Roskilde, Denmark) coated with 50 µg/ml nArt v 1. Bound phages were eluted by 10-min incubation with 1 µg/ml trypsin. Exponentially growing TG1 bacteria (McCafferty et al., 1990) in 2× TY medium were infected with eluted phages and plated on 2× TY containing 1% glucose and 100 µg/ml ampicillin. The

next day, cells were abraded and grown to an optical density at 600 nm (OD₆₀₀) of 0.4 in 2× TY medium containing 1% glucose and 100 µg/ml ampicillin before hyperphage M13K07 ΔpIII (Progen Biotechnik, Heidelberg, Germany) infection at a bacteria/phage ratio of 1:10 for 30 min at 37°C (Rondot et al., 2001). Infected cells were harvested, resuspended in 2× TY medium containing 100 µg/ml ampicillin and 30 µg/ml kanamycin, and incubated overnight at 30°C. After centrifugation for 10 min at 10 800 g and 4°C, rescued phages in the supernatant were precipitated by addition of a 1/4 culture volume of 20% polyethylene glycol 8000 and 2.5 M NaCl, harvested, resuspended in pre-cooled PBS, and used for subsequent panning and selection rounds.

ELISA screening for positive clones was performed after four rounds of panning. Briefly, Nunc-Immuno MaxiSorb microtiter plates (Nunc, Roskilde, Denmark) were coated with 0.5 µg of nArt v 1 or rArt v 1. Single infected TG1 colonies were grown and infected with hyperphage as described above. Supernatants were used for antigen incubation and horseradish peroxidase-labeled anti-M13 monoclonal conjugates (GE Healthcare, Chalfont St. Giles, UK) served for detection using 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) as substrate. Absorption was measured at 405 nm using a Dynatech MR 600 ELISA reader (Dynex Technologies, Chantilly, VA, USA).

Cloning of scFv

Phagemids containing scFv antibodies were cloned into pDAP2/S (Kerschbaumer et al., 1996), adding a PstI site at the 5'-end and a NotI site at the 3'-end of the coding regions using the primers 5'-GAG AGA GAC TGC AGG CCG AGG TGC AGC TGG TGG AG-3' and 5'-GAG AGA GAG GAA TCG TCA TCT GCG GCC GC-3'. Sequencing was performed on an ABI PRISM™ Big Dye™ Terminator Cycle Sequencing system (Applied Biosystems, Foster City, CA, USA).

Production of scFv-AP

TG1 cells transformed with engineered plasmids were grown to an OD₆₀₀ of 0.4 at 30°C. Pre-cultures were diluted 1:100 in M9ZB and grown to an OD₆₀₀ of ≤0.5 at 30°C. Cells were harvested and resuspended in an equal volume of modified M9ZB lacking glucose and containing 1% (v/v) glycerol. Expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside, and cells were harvested after 68 h at 16°C. Proteins in the culture supernatant were precipitated with 70% (NH₄)₂SO₄ and dialyzed to 10 mM Tris, pH 7.5, 140 mM NaCl, and 10 mM imidazole for IMAC performed on HisTrap HP columns using the ÄKTA prime system (GE Healthcare). Histidine-tagged protein was eluted using a stepwise gradient (10%, 15%, and 20%) to 10 mM Tris, pH 7.5, 140 mM NaCl, and 500 mM imidazole. Fractions containing pure and AP active protein were pooled and diafiltrated to 10 mM Tris, pH 7.5, 140 mM NaCl using 10-kDa Amicon filter devices (Millipore, Bedford, MA, USA).

Immunological and biochemical characterizations

Pollen extracts, bacterial lysates, and purified allergens were analyzed by SDS-PAGE. For immunoblot analysis, proteins were separated by gel electrophoresis and electroblotted onto nitrocellulose Protan membranes (Schleicher & Schuell, Dassel, Germany). Membranes were blocked with 25 mM Tris-HCl, pH 7.5, 0.5% bovine serum albumin (w/v), 150 mM NaCl, and 0.5% Tween 20 and incubated with several dilutions of scFv-AP, anti-defensin mAb, or polyclonal Art v 1-specific rabbit sera (Himly et al., 2003), followed by incubation with AP-conjugated rabbit anti-mouse IgG and IgM (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or AP conjugated goat anti-rabbit

IgG, respectively. Detection was performed with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate substrates. IgE immunoblotting was performed according to previously published protocols (Himly et al., 2003). In addition, the antibodies produced were analyzed by ELISA as described elsewhere (Wallner et al., 2007). Carbohydrates were detected by staining with periodic acid Schiff's (PAS) reagent (Himly et al., 2003).

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