

Ragweed Pollen Allergy: Burden, Characteristics, and Management of an Imported Allergen Source in Europe

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Abstract

Ambrosia artemisiifolia, also known as common or short ragweed, is an invasive annual flowering herbaceous plant that has its origin in North America. Nowadays, ragweed can be found in many areas worldwide. Ragweed pollen is known for its high potential to cause type I allergic reactions in late summer and autumn and represents a major health problem in America and several countries in Europe. Climate change and urbanization, as well as long distance transport capacity, enhance the spread of ragweed pollen. Therefore ragweed is becoming domestic in non-invaded areas which in turn will increase the sensitization rate. So far 11 ragweed allergens have been described and, according to IgE reactivity, Amb a 1 and Amb a 11 seem to be major allergens. Sensitization rates of the other allergens vary between 10 and 50%. Most of the allergens have already been recombinantly produced, but most of them have not been characterized regarding their allergenic activity, therefore no conclusion on the clinical relevance of all the allergens can be made, which is important and necessary for an accurate diagnosis. Phar-

macotherapy is the most common treatment for ragweed pollen allergy but fails to impact on the course of allergy. Allergen-specific immunotherapy (AIT) is the only causative and disease-modifying treatment of allergy with long-lasting effects, but currently it is based on the administration of ragweed pollen extract or Amb a 1 only. In order to improve ragweed pollen AIT, new strategies are required with higher efficacy and safety.

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Introduction

Ambrosia (ragweed) is an invasive annual flowering herbaceous plant from the family Asteraceae which originated from North America. About 40 species are known and *Ambrosia artemisiifolia* (common or short ragweed) and *A. trifida* (giant ragweed) are the most common species [1]. Among all Ambrosia species, *A. artemisiifolia* is the most prominent and invasive, being a major cause of allergy in late summer worldwide. The plant is characterized by a bushy-branched stem with pinnately lobed leaves (Fig. 1).

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Fig. 1. Appearance of *A. artemisiifolia*. The height of the plant varies from 10 cm to 2.5 m according to environmental conditions. Stems are sparsely to densely pubescent with relatively long hairs. The pinnately lobed leaves are opposite towards the stem base, but alternate towards the stem tip.

In Europe, the first observation of *A. artemisiifolia* was in the mid of the 19th century [2] but an explosive expansion of ragweed occurred after 1900 due to the import of contaminated grains and seeds from North America, and this invasion event is still ongoing [1].

The most invaded areas with *A. artemisiifolia* and therefore the most important sources of ragweed pollen in Europe can be found in a few particular areas, which are the Pannonian Plain, especially Hungary and neighboring countries such as Romania, the Rhône Valley in France, parts of northern Italy and, just recently reported, Ukraine [3–7]. Beside North America and Europe, ragweed plants can also be found on other continents, including Asia, Australia, Africa, and South America [1, 8], which confirms the huge invasive ability of ragweed (Fig. 2).

In North America, ragweed has long been recognized as a major health problem. Allergic rhinitis and asthma are the main allergic diseases that have been associated with exposure to ragweed pollen, while skin allergic reactions are less common. In the 1930s ragweed was identified as the major cause of hay fever and asthma, and in the 1940s the first eradication program was initiated for ragweed using herbicide [9]. Currently, the sensitization rates against ragweed pollen in the USA range between 15 and 26% of the common population [10, 11]. In Europe, one of the first reports about ragweed pollen sensitization was published in the 1980s [12, 13]. The sensitization rates among atopic patients can vary tremendously depending on the country. Sensitization rates of 60, 47, and 70% could be observed in Hungary [14], France, mainly

the Rhône Valley [15], and in northern Italy [16], respectively. In other parts of Europe, such as Spain or the UK, no significant ragweed sensitization could be determined [17]. The situation in Asia seems to be different than in America and Europe. Although the ragweed plant is also now established in Japan, South Korea, and certain parts of China [1, 18], the sensitization rate seems to be low, at around 5% [18–20].

Ragweed pollen allergy represents a major health issue and this may be due to some characteristics of the ragweed plant or pollen. One main characteristic is the high pollen production of the ragweed plant and the allergenic potency of the ragweed pollen itself. One single ragweed plant can release up to one billion pollen grains per season [21]. Studies have shown that high pollen exposure or the increase of pollen counts over a certain period of time leads to a strong increase of the sensitization rate and the occurrence of symptoms [13, 16]. It is important to underline that even low exposure, meaning as little as 10 pollen grains per cubic meter of air, can trigger an allergic reaction [22, 23]. The diameter of a ragweed pollen grain is only 15–25 μm and the pollen surface is covered in short spines (Fig. 3). Ragweed pollen grains can be transported several hundreds to thousands of kilometers by air and can cause allergy symptoms in areas where the ragweed plant is not widespread [6, 24, 25]. In Denmark for instance, the occurrence of ragweed is moderate compared to Hungary, but sometimes peaks in pollen counts can be observed. Investigation has revealed that this additional pollen load had its origin in Ukraine and Romania, which are more than 1,000 kilometers away [26].

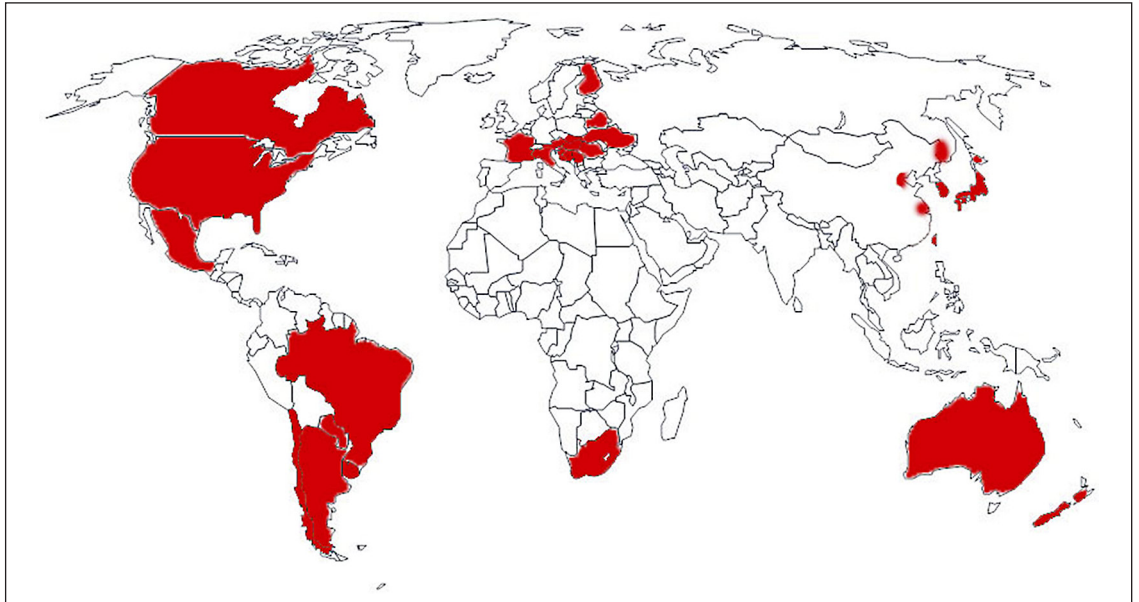


Fig. 2. Worldwide distribution of ragweed. Ragweed occurrence has been reported in different countries (red) in North America (Canada, the USA, Mexico), South America (Brazil, Uruguay, Paraguay, Argentina, Chile), Europe (Hungary and neighboring countries, Italy, France, Finland), Africa (South Africa), Asia (Japan, South Korea, China), Australia and New Zealand.

It has been suggested that *de novo* sensitization in adults is quite frequent [16, 27] and ragweed pollen induces asthma much more frequently than other pollens [28]. For example, 23.7% of sensitized ragweed pollen-allergic patients showed asthma symptoms [14], another indicator for the allergenic potency of ragweed. The high sensitization rates and high percentage of asthma incidences may be explained by some contents in the ragweed pollen grains, such as a high concentration of NADPH oxidase and/or serine and cysteine proteases [29–31].

Environmental factors such as temperature and CO₂ concentrations have great influences on pollen production and therefore on the allergen amount [32–34]. These two environmental factors are increasing due to climate change and urbanization. Prediction models indicate that ragweed will be widely spread and become domestic in large parts of Europe [3, 35, 36].

Due to their high prevalence and severe symptoms, ragweed pollen-induced allergic rhinitis and asthma may significantly affect quality of life, with an impact on attendance and performance at school or the workplace, leading to considerable healthcare costs and a high economic burden. In contrast to the current situation, ragweed pollen allergy will become a significant health issue in Europe which will not be restricted to only particular areas.

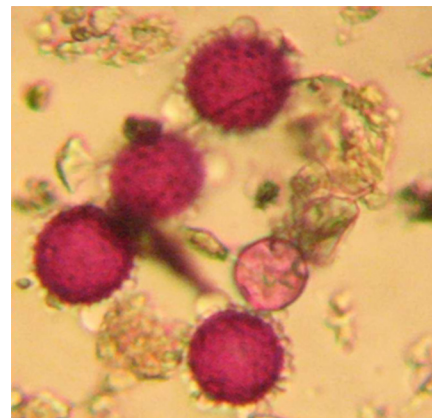


Fig. 3. *A. artemisiifolia* pollen. Ambrosia pollen dyed with fuchsin (red) and visualized on an Optika B500 microscope at $\times 400$ magnification.

Molecular Characterization of Ragweed Pollen Allergens

Ragweed pollen allergens were studied for over half a century, with changing nomenclature over time, and still new allergens are being identified. There are currently 11 ragweed pollen allergens included in the IUIS database

Table 1. Synoptic presentation of IUIS-recognized ragweed allergens [18, 38, 44, 52, 53, 74]

Allergen	IgE sensitization rate	MW, kDa	Description
Amb a 1	90–95%	38	Pectate-lyase (enzyme), with 5 main isoforms, cross-reactive with Art v 6
Amb a 2	Regrouped as Amb a 1.05 isoform		
Amb a 3	30–50%	11	Plastocyanin (copper-binding protein)
Amb a 4	20–40%	30	Defensin, cross-reactive with Art v 1
Amb a 5	10–15%	5	Cross-reactive with other ragweed proteins – Amb p 5, Amb t 5
Amb a 6	20–35%	10	Non-specific lipid transfer protein, panallergen, cross-reactive especially with food allergens
Amb a 7	15–20%	12	Plastocyanin (copper-binding protein), partly known sequence
Amb a 8	20–35%	14	Profilin, important panallergen
Amb a 9	10–15%	10	Polcalcin, with 2 EF-hand domains (Bet v 4-like), panallergen
Amb a 10	10–15%	18	Polcalcin, with 3 EF-hand domains, panallergen
Amb a 11	50–66%	37	Cystein-protease (enzyme), more than potential 20 isoforms and glycoforms
Amb a 12	41–68%	48	Enolase

(www.allergen.org). Among these, 2 are described as major allergens, while the others are considered as minor allergens (Table 1) [37].

Major Ragweed Pollen Allergens

Amb a 1 is a major allergen belonging to the pectate lyase protein family. Such enzymes are important for pollen growth by degrading pectines [38]. More than 90% of ragweed-allergic patients are sensitized to this major allergen [39]. Furthermore, Amb a 1 is an acidic non-glycosylated protein with 397 amino acids (AA) and has a molecular weight of approximately 38 kDa [40]. Disulfide bonds can be located between C54–C71 and C211–C235, and potentially between C391 and C397 [41].

Currently, 5 Amb a 1 isoforms are described in the WHO/IUIS allergen nomenclature database [37] with identities between 63 and 86% (see online suppl. Table S1a; see www.karger.com/doi/10.1159/000487997 for all online suppl. material). Immunological characterization of these 5 isoforms revealed different IgE-binding capacities, indicating that Amb a 1.01 has the highest allergenic activity [42].

No structural data such as X-ray crystallography are available for Amb a 1, but a homology model based on the crystal structure of the major cedar pollen allergen Jun a 1 (*Juniperus ashei*) indicates a core structure consisting primarily of a parallel beta-helix [41, 43] (Fig. 4). During purification, Amb a 1 can be proteolytically cleaved into 2 units, a 26-kDa alpha (AA 181–396) and a 12-kDa beta (AA 26–180) subchain with different humoral (IgE) and cellular (T-cell) reactivity in sensitized individuals [40].

The alpha-subchain showed reduced IgE reactivity but comparable T-cell reactivity with the native allergen, whereas the beta-subchain showed a comparable IgE reactivity but reduced T-cell reactivity [41].

The expression of Amb a 1.01, 1.02, and 1.03 isoallergens could be upregulated under certain environmental situations, such as elevated CO₂ or drought stress, which has been shown in greenhouse experiments [38]. Also, environmental factors such as temperature, humidity, and light had a significant influence on Amb a 1 content [32]. Another study revealed that fumigation of ragweed plant with a mixture of elevated NO₂ (approx. 90%) and NO increased the Amb a 1 allergen expression at least 1.5-fold. This may explain why pollen collected in polluted areas has a higher allergenicity [44].

Amb a 1 shows a sequence homology of 58% with the mugwort allergen Art v 6 (see online suppl. Table S1a). Furthermore, Amb a 1 shares some sequence homology with allergens from the Cupressaceae family, such as Cry j 1 (Japanese cedar), Jun a 1 (mountain cedar), Cup a 1 (Arizona cypress), which ranges between 44 and 45%. Amb a 1 shares the highest sequence homology (67.8%) with the pectate lyase from *Helianthus annuus* (sunflower; see online suppl. Table S1a). Nevertheless, sunflower sensitization is rare, which may be due to the characteristics of sunflower pollen. Sunflower pollen is larger than ragweed pollen and therefore its wind dispersion is very limited. Exposure to sunflower pollen occurs only locally. Still, the exposed population, especially agricultural workers, can become sensitized to sunflower pollen and reach a sensitization rate of about 20% [45, 46].

A comparison between Amb a 1 and the closely related weed allergen Art v 6 revealed that both allergens are

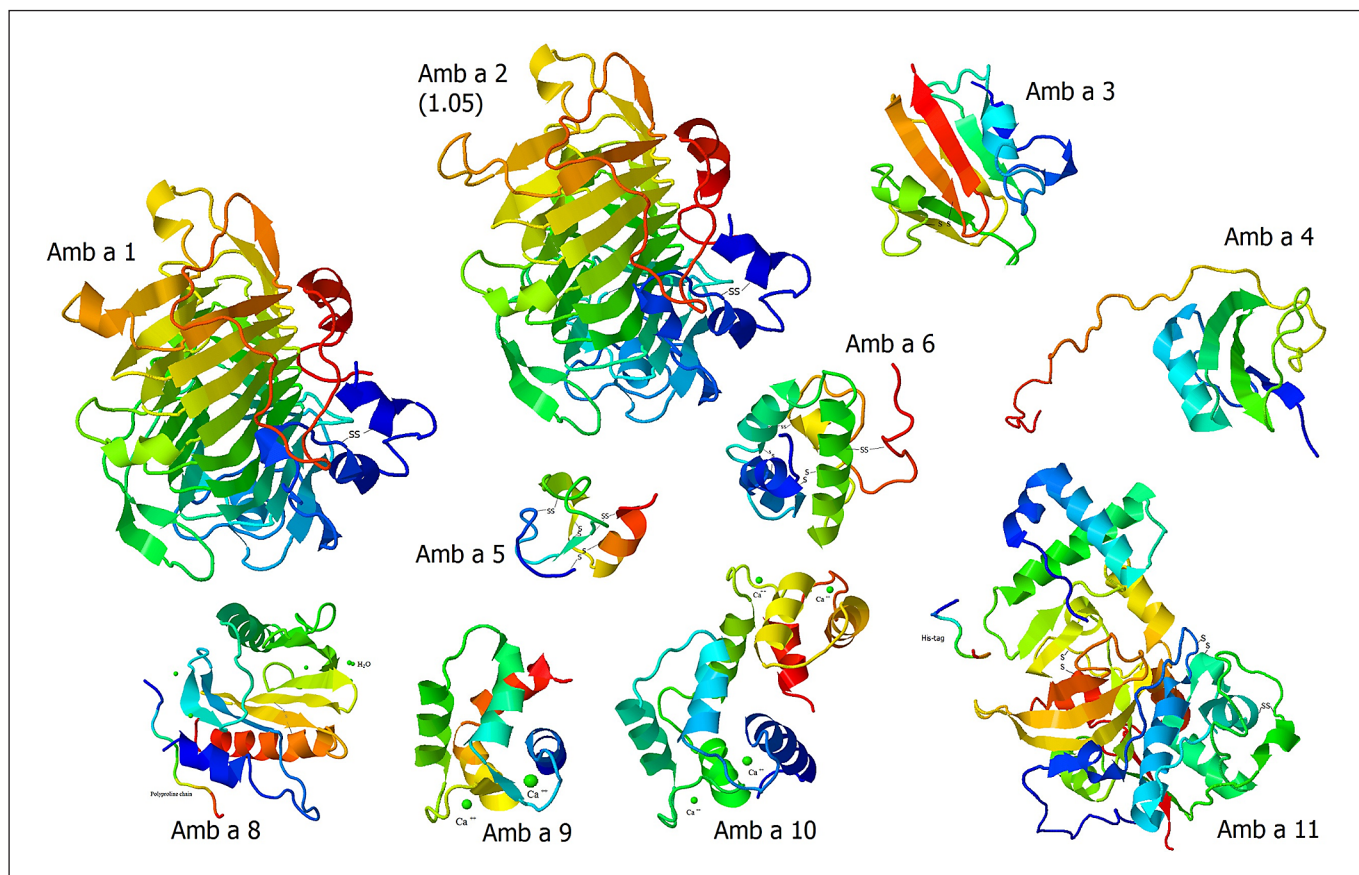


Fig. 4. Structure of ragweed allergens. Ribbon representations of ragweed allergen models were generated by the SWISS-MODEL online tool (<https://swissmodel.expasy.org/>) using similarity with known structures. Jmol software was used to display the structure

in a color gradient, indicating the N-terminal in red and the C-terminal in blue. Amb a 8 and 11 structures were determined directly by X-ray crystallography. Amb a 7 is missing because no full sequence is available.

cross-reactive [47], but Amb a 1 possesses more IgE epitopes and is a better T-cell stimulant [48]. Another study investigating the cross-reactivity between Amb a 1 and homolog pectate lyase allergens from the botanically unrelated Cupressaceae family (Cry j 1, Jun a 1, and Cup a 1) revealed no significant cross-reactivity between these 2 families [18].

Peripheral blood mononuclear cells from ragweed pollen-allergic individuals were used to map T-cell epitopes and 6 dominant epitopes were identified at position AA176–191, AA200–215, AA280–295, AA304–319, AA320–335, and AA344–359 [49]. These findings may be useful for new immunotherapy strategies.

Amb a 1 (isoform Amb a 1.0301) has been expressed in recombinant form, first in *Escherichia coli* as His-tagged protein. However, this recombinant allergen showed a very low IgE binding activity, maybe due to mis-

folding [41, 50]. Recently, Amb a 1.0301 has been expressed in *Pichia pastoris* with an IgE reactivity comparable with the native form [42].

Amb a 2, designated now as Amb a 1.05, is an Amb a 1 isoform showing 67% sequence identity (see online suppl. Table S1a) with Amb a 1.01. Amb a 2 is an allergen with 397 AA and has a molecular weight of 38 kDa [51].

Recently, Amb a 11, a cysteine protease, has been suggested as a novel major allergen with a sensitization rate of up to 66% [52]. This allergen has a molecular weight of approximately 37 kDa in its mature form with 386 AA. Since Amb a 1 has a nearly identical size, it can only be detected and visualized on a 2D blot analysis. There is an N-glycosylation site at the 19th position with variable glycan ligands and 3 disulfide bridges stabilizing the protein [52]. Detailed mass spectrometry analysis of purified natural Amb a 11 revealed that more

than 20 potential isoforms and glycoforms may exist [52].

The crystal structure of Amb a 11 was identified by using a recombinant proform. The structure of pro rAmb a 11 is typical of a C1A cysteine protease consisting of 6 alpha-helices and a beta-sheet formed from 6 antiparallel beta-strands in its mature form (Fig. 4) [53].

Regarding the allergenic potency of the molecule, sensitization with mature rAmb a 11 in mice induced strong allergic inflammation after challenge with ragweed pollen extract. In detail, the mice showed airway hyperresponsiveness, high levels of eosinophils and innate lymphoid type 2 cells (ILC2) in bronchoalveolar lavage fluid, as well as Amb a 11-specific IgE and IgG₁ responses after challenge with ragweed pollen extract. However, sensitization with pro-rAmb a 11 or E64-inhibited rAmb a 11 resulted in lower allergic responses [53]. These effects correlate with reports on cysteine protease allergen-induced disruption of airway epithelium, as well as with protease-enhanced activation of immune cells and initiation of Th2-type inflammation [54].

Sequence alignment with other allergens showed that Amb a 11 shares homology with other major allergens belonging to the same protease family, such as Act d 1 from kiwi fruit (36.9%), Ana c 2 from pineapple (34.1%), and Der f/Der p 1 from dust mites (27.3/23.5%; see online suppl. Table S1i).

Comparing the IgE sensitization profile in 92 American and European patients revealed 4 distinct patterns, which are Amb a 1 alone (40% of the patients), multiple allergens including the major allergens (15%), mainly Amb a 1 and Amb a 11 (30%), or mainly Amb a 11 (15%) [52]. This indicates that Amb a 11 should also be included in diagnosis and treatment.

Minor Ragweed Pollen Allergens

Many minor allergens can be assigned to panallergens, which are proteins sharing highly homologous sequences, structures, and functions. These similarities can lead to IgE cross-reactions. Protein families, such as profilins, polcalcins, and non-specific lipid transfer proteins (nsLTP) are known panallergens and many minor ragweed allergens correspond to these families.

Amb a 8 belongs to the profilin family with a molecular weight of approximately 14 kDa (133 AA) and 2 isoforms have been identified [55, 56]. Profilins are small proteins which regulate actin polymerization and depolymerization during pollen growth [57]. Profilins have

been discovered as highly cross-reactive allergens in birch [58, 59] and were identified as actin-binding proteins in plants soon thereafter [60]. The sensitization rate is approximately 26% in ragweed pollen-allergic patients [56].

The Amb a 8 molecule has a disulfide bond between C95 and C117, a poly-proline region and an actin binding site. Further characterization revealed the structure showing 2 terminal alpha-helices, 1 short alpha-helix, and a beta-hairpin that sandwich a central 5-stranded antiparallel beta-sheet [61] (Fig. 4).

In allergic patients, profilins could be responsible for cross-reactivity with other food and pollen allergens, such as Cor a 2 (hazelnut), Mal d 4 (apple), Dau c 4 (carrot), Mus a 1 (banana), Pru p 4 (peach), Hel a 2 (sunflower), Phl p 12 (timothy grass), Bet v 2 (birch), Ole e 2 (olive), Cyn d 12 (Bermuda grass), and with many other allergens. All these cross-reactive allergens showed a sequence identity of over 65% (see online suppl. Table S1c). In addition, Amb a 8.0101 shares 89.5% of its AA sequence with Art v 4.0101, displaying comparable cross-reactivity within the same family due to structural similarities between the 2 proteins [61]. There is a close relationship between ragweed and sunflower proteins, as the highest homology outside the species is recorded between ragweed and sunflower, mainly for profilin (94% sequence homology, see online suppl. Table S1f) but also for pectate lyase (68.4% sequence homology, see online suppl. Table S1a). The transcript for Amb a 8.1 was downregulated in elevated CO₂ conditions in a greenhouse experiment [38].

Amb a 9 and Amb a 10 belong to polcalcins, a family of calcium-binding proteins (CBP) containing EF-hand domains. CBPs play an essential role in calcium signaling during pollen tube growth [62].

Amb a 9 has a molecular weight of 9 kDa with a length of 82 AA and Amb a 10 has a molecular weight of 18 kDa with a length of 160 AA, and the sensitization rate in ragweed pollen-allergic patients is approximately 10–15% for both allergens [56, 63]. Amb a 9 has 2 EF-hand domains and belongs to the Bet v 4-like polcalcins group, while Amb a 10 has 3 such domains. Structural analysis performed with circular dichroism revealed a primarily alpha-helical secondary structure (Fig. 4). Calcium binding may alter the conformation of the allergen significantly, which may then influence the IgE binding capacity [64]. The absence of calcium did not alter the structure significantly while calcium was necessary for IgE binding [56]. Sequence alignment analysis showed shared sequence homology with other allergens from the same protein family, such as Art v 5, Syr v 3, Bet v 4, and Ole e

3 for Amb a 9 and for Amb a 10 similarity with Ole e 8, Cyn d 7, and Phl p 7 (see online suppl. Table S1g, h) and variable cross-reactivity with other polcalcins can be observed [56]. Expression of Amb a 9 and 10 was upregulated in drought stress and elevated CO₂ conditions in a greenhouse experiment [38].

Amb a 6 is a basic 10-kDa type I nsLTP with a sensitization rate of 21% among ragweed pollen-allergic patients [65]. This allergen is expressed as a promolecule with 188 AA residues including an N-terminal 25 AA signal peptide [66].

A structural model generated by the SWISS-MODEL online tool (<https://swissmodel.expasy.org/>) indicates that Amb a 6 contains 4 alpha-helices cross-linked with 4 disulfide bridges that stabilize a hydrophobic inner cavity (Fig. 4). Although nsLTPs are a family of panallergens commonly found in fruits and nuts, sequence homology of Amb a 6 with other nsLTPs is limited, indicated by an identity score of below 40% (see online suppl. Table S1e). However, cross-reactivity among pollen LTPs could not be observed and cross-reactivity between nsLTPs from foods and pollens is limited [67].

Amb a 3 is a highly basic protein belonging to the plastocyanin family. It has a single-copper ion-binding site, which is involved in the electron transport chain in plants. Amb a 3 has a molecular weight of approximately 11 kDa (101 AA) and can be N-glycosylated on position AA 41 and O-glycosylated on position AA 84 [68]. The sensitization rate has been reported to be between 30 and 50% [69] classifying Amb a 3 as a minor allergen. Sequence homology analysis showed no homology with other allergens but some similarities with other plant proteins such as the sunflower, *H. annuus* (63%; see online suppl. Table S1b).

Amb a 7 is another minor allergen belonging to the plastocyanin family. Not much is known about this allergen. In 1991, it was reported that Amb a 7 was isolated and purified from ragweed extract via chromatography methods. N-terminal sequencing of this approximate 12-kDa protein revealed a partial sequence homology with a 96 residue cucumber “cusacyanin” (57% identity) and a 15–20% IgE reactivity in ragweed pollen-allergic patients was determined [70].

Amb a 4 belongs to the defensin-like protein family with a molecular weight of approximately 30 kDa. Further characterization identified Amb a 4 as a glycoprotein with a proline-rich C-terminal region and 4 intramolecular disulfide bonds. A sensitization rate of 20–39% among ragweed pollen-allergic patients was determined [71].

A structure model generated by the SWISS-MODEL online tool shows a molecule with an alpha-helix, 3 anti-

parallel beta-strands, and a proline-rich region (Fig. 4). This structure composition seems to be characteristic for allergens belonging to this protein family [72].

Sequence alignment analysis showed high homology (over 60%) between Amb a 4, Par h 1 (*Parthenium hysterophorus*, feverfew), and a defensin-like protein originated from *H. annuus*. Similarities with different species of *Artemisia* were between 50 and 54% (see online suppl. Table S1c). This observation has also been made by other studies [71, 72].

Patients showing IgE reactivity to Amb a 4 often also react to Art v 1. Whether this reaction is due to cross-reactivity or co-sensitization remains unclear [71, 72].

Amb a 5 is a small molecule with a molecular weight of 5 kDa and is 45 AA long [73, 74]. The sensitization rate is reported to be around 10% [75]. Structural analysis of Amb a 5 revealed a molecule with a C-terminal alpha-helix, a short stretch of triple stranded antiparallel beta-sheets and several loops. This structure is stabilized by 4 disulfide bonds [76] (Fig. 4). Sequence analysis showed that Amb a 5 only shares similarities with proteins from the same genus (see online suppl. Table S1d).

A very recently identified allergen mentioned in the IUIS database, Amb a 12, is an enolase with a molecular weight of approximately 48 kDa, with a homologous structure to Hev b 9 and a significant prevalence of IgE reactivity, reported as being from 41 to 68%. A number of proteins with enolase activity and molecular weights from 42 to 51 kDa have been identified as potential allergens in recent studies [37, 44, 77].

Potential New Ragweed Allergens

A study using transcriptomic and immunoproteomic techniques to screen for potential new allergens using 22 sera from ragweed pollen-allergic patients indicated a 68 and 41% sensitization rate to ragweed polygalacturonase (existing in 2 isoforms) and enolase, respectively. Seven novel candidate allergens are proposed, including the 2 already mentioned and a form of carbonic anhydrase, galactose-oxidase (2 isoforms), UDP-glucose pyrophosphorylase, GDP dissociation inhibitor, and pathogenesis-related 17 (PR-17) protein. The last 2 were designated as the most promising candidates for new allergens. A number of plastocyanin-like proteins were also identified, with IgE reactivity between 10 and 20% [77].

Another proposed minor allergen is Amb a CPI, a cysteine protease inhibitor present in ragweed pollen [78], with homology to other plant allergens found in *Acti-*

nidia deliciosa [79]. Elevated ragweed plant exposure to ozone can induce a higher level of expression for Amb a CPI and for the major allergen Amb a 1, as was revealed by transcriptomic assays [80].

Therapeutic Approaches in Ragweed Allergy

There are no consistent data regarding the relationship between ragweed pollen exposure and the severity of allergic reactions. However, the treatment should be tailored in accordance with the level of symptoms, regardless of the level of exposure (lower or higher). There are 3 major therapeutic approaches for allergic rhinitis and asthma: (1) allergen avoidance and reduced exposure to triggers, (2) symptomatic medication, and (3) allergen-specific immunotherapy (AIT).

Allergen Avoidance

Allergen avoidance strategies can effectively improve allergic symptoms. However, unlike in food allergy, complete avoidance may be difficult to achieve, especially for pollen allergens. Exposure to pollen can be minimized by using air conditioners to filter the air, keeping windows closed, especially during the day, and reducing the time spent outdoors during peak pollen season. In order to inform people about pollen conditions, attempts have been made to develop regional specific pollen counts in the atmosphere or to create forecasting models [7].

The only feasible way to avoid ragweed pollen is to limit ragweed spread, which is currently an important challenge in Europe. Different methods, such as herbicides, mowing, weeding, competition vegetation, crop rotation, disking, grazing, milling and plowing, singeing, or mulching, have been employed with varying effectiveness in order to control ragweed expansion [81].

Symptomatic Treatment Options

Symptomatic treatment is widely used to manage ragweed pollen-induced allergic rhinoconjunctivitis. It mainly consists of oral non-sedating H1-antihistamines and intranasal glucocorticoids, according to the Allergic Rhinitis and Its Impact on Asthma (ARIA) guidelines (<http://www.euforea.eu/about-us/aria.html>). In addition, mast cell stabilizers, leukotriene antagonists, and anti-inflammatory eye drops can be taken. Combined ap-

proaches with multiple therapeutic agents are frequently administered.

For ragweed pollen-induced allergic asthma, the main therapeutic classes are inhaled corticosteroids (ICS) and β_2 -agonists. According to the Global Initiative for Asthma (GINA) guideline, patients with purely seasonal allergic asthma, such as ragweed pollen-induced allergic asthma, ICS should be taken as soon as symptoms appear, and continued for 4 weeks after the end of the pollen season [82].

Future therapeutic options, such as selective antagonists for H3 histamine, DP2 and leukotriene B4 receptors, or immunomodulators which target Toll-like receptors (TLRs), aim to ensure increased efficiency, easier administration, and fewer side effects [83–86]. Even though the effectiveness of symptomatic medication is certified by randomized controlled clinical trials, pharmacotherapy often cannot adequately control symptoms [87] and it may induce side effects. In the case of ragweed pollen allergy, symptom control can be reached in less than 50% of the patients using conventional therapies [88]. Moreover, symptomatic treatment cannot impact the underlying immune mechanisms of allergy, and consequently cannot change the course of disease [89]. The pros and cons of common symptomatic medication and of AIT are listed in Table 2.

Allergen-Specific Immunotherapy

AIT is the only disease-modifying treatment, with long-lasting effects even after its discontinuation, and is able to stop the atopic march [90, 91]. AIT is indicated for the treatment of moderate-to-severe intermittent or persistent symptoms of allergic rhinoconjunctivitis, especially in patients with a poor response to pharmacotherapy, according to the ARIA guidelines. Regarding asthma, the current ARIA, ICON (International Consensus on Allergic Immunotherapy), and GINA guidelines give AIT a conditional recommendation, due to moderate or low quality of evidence.

Ragweed pollen AIT has been in use for over 100 years. The first successful trial of ragweed pollen subcutaneous immunotherapy (SCIT) was conducted in 1913, and used aqueous ragweed pollen extracts [92]. Another study from 1935 showed that transfer of serum from patients who had received treatment with ragweed extract confer protection during the ragweed pollen season to immunotherapy-naïve patients [93]. A study on sera from patients treated with ragweed extract for up to 20 years was con-

Table 2. Pros and cons of common symptomatic medication versus AIT for allergic rhinitis

	Advantages	Disadvantages
Antihistamines	Immediate effects Good symptom control Long-lasting effects (up to 24 h)	Somnolence (more for 1st generation) Confusion (more for 1st generation) Moodiness (more for 1st generation) Dizziness (more for 1st generation) Xerostomia
Glucocorticoids	Good symptom control	Late onset of action Long period until reaching symptom control Epistaxis Nasal irritation Systemic disorders (Cushing syndrome)
Immunotherapy	The only disease-modifying treatment	Long duration of treatment Possible immediate or delayed reactions

ducted in 1964, showing a progressive decline in the concentration of sensitizing antibodies [94]. The first double-blind randomized clinical trial on the efficacy of a multi-allergen mix for ragweed pollen allergy was conducted by Lowell and Franklin [95].

Allergen extract-based AIT is not yet widespread due to several reasons: the vaccination schedule may be inconvenient, requiring at least 3 years of therapy, the costs may be high, and there is always the risk of severe side effects. The main concerns of using natural allergen extracts are their quality, more precisely the presence of contaminants or undefined components that may promote allergic immune responses, and variable amounts of relevant allergens and thus differences in immunogenicity [96]. Analysis of different extracts used for the diagnosis and treatment of ragweed pollen allergy revealed a tremendous variation in allergen content and allergen concentration, which could significantly impact the treatment by reduced efficiency of such agents. The variation is underlined especially when measuring the relative potency of ragweed pollen extracts in Amb a 1 units, emphasizing the correlation between major allergen contents and the extract's biological potency [97].

The first improvement of ragweed AIT was the use of adjuvants (e.g., aluminum hydroxide, calcium phosphate, or tyrosine) and the chemical modification of extracts (allergoids), which lowered the risk of systemic side effects and enabled the faster achievement of the maintenance dose in comparison with aqueous extracts [98]. New adjuvants, such as monophosphoryl lipid A, the non-pyrogenic component of *Salmonella minnesota* LPS, combined with ragweed allergoid adsorbed to L-tyrosine, in

order to boost its immunogenicity, are currently available [99, 100]. Another approach of AIT improvement is the additional treatment with omalizumab, a monoclonal anti-IgE antibody [101], which is an off-label use [102]. Omalizumab blocks IgE binding to FcεR1 on the surface of effector cells (e.g., mast cells, basophils) [103], limiting the effects of allergen exposure mediated by these cells. The rationale for combining AIT and omalizumab is based on their complementary action which can increase AIT safety. There are clinical data showing that omalizumab pretreatment can reduce the side effects of subcutaneous rush immunotherapy for ragweed pollen-induced allergic rhinitis [104].

Currently, the challenge to develop better products for AIT remains, in terms of safety, efficacy, and cost-benefit ratio, in order to increase patient compliance, without interfering with the immunomodulatory activity. The strategies addressing this challenge include the introduction of other administration routes, shorter build-up schedules of administration, and new forms of treatment based on recombinant allergens, hypoallergenic allergen derivatives, and allergen-derived peptides [105–107].

While SCIT has traditionally been used for administration of AIT, sublingual immunotherapy (SLIT) has been gaining interest, since its introduction in 1986, due to the potentially lower risk of severe side effects [108]. Most SLIT products currently on the market are solutions, but SLIT tablets entered the North American market in 2014, and are being prepared for approval in Europe. Several randomized, controlled clinical trials have been conducted in order to investigate SLIT efficacy and safety for ragweed pollen allergy [109–111]. At the mo-

ment, SCIT is reasonably well standardized regarding administration protocols [112], whereas protocols for SLIT are less defined. Direct comparison studies between the two administration routes would be needed to compare the two approaches [113, 114]. One obstacle of SLIT is the low compliance. A large-scale “real-life” study conducted in 2014 comparing SCIT and SLIT compliance, revealed that overall compliance for at least 3 years of treatment is less than 50%, with a better compliance within the SCIT group [115].

Taking into account that most of the ragweed allergens exist in recombinant form, they open the field for new AIT strategies based on the production of hypoallergenic variants. These variants can be generated by introducing mutations and deletions around the B-cell epitope (IgE binding) areas, fragmentation, oligomerization, or fusion of allergen variants (chimeras). These recombinant proteins should not be IgE reactive but still be immunogenic, in order to allow higher dose administration without inducing side effects in comparison to wild-type molecules [105]. These candidates are able to replace allergen extracts and can be produced in unlimited amounts, with highly standardized quality and exact physicochemical and immunological characteristics. Products based on recombinant hypoallergens are currently under development and others have already been evaluated in phase II and III clinical trials [116–118].

An innovative approach in ragweed pollen AIT could be the development of hypoallergenic derivatives based on allergen-derived peptides reassembled into protein constructs [119] or the combination of a carrier molecule with these peptides, which will provide enhanced stability and immunogenicity, without IgE reactivity [120]. Promising results have been attained with these carrier molecules, among which are bacteriophage Qb-derived virus-like particles [121], hepatitis B virus pre-S protein [122–124], and polyethylene glycol [125].

Some attempts have been made to find novel candidates for a therapeutic approach in ragweed allergy. A recent study generated and preclinically characterized the immunogenic domains of Amb a 1 and Art v 6, envisaging their use for immunotherapy. The study revealed that preservation of T-cell epitopes together with deletion of IgE reactive areas of Amb a 1 and Art v 6 can modulate the immunologic characteristics of the allergen, a property that makes the new molecules suitable candidates for AIT [126].

It has been hypothesized that the T-cell epitopes presented by HLA-DP or HLA-DQ molecules, as is the case with Amb a 1, might sensitize a large part of the population [127]. The use of allergens combined with TLR li-

gands, which modifies the activity of antigen-presenting cells, has been proposed as a new approach for shifting from a Th2-polarized towards Th1-oriented immune response pattern [128]. Amb a 1-immunostimulatory phosphorothioate oligonucleotide conjugates have been produced. This conjugate contains a short synthetic DNA sequence that binds to TLR9 on plasmacytoid dendritic cells, thereby activating the innate immune system. This product has shown good short-term and long-term effects in subjects with moderate-severe ragweed pollen allergy, presenting low allergenicity and inducing an enhanced Th1-type response. The effects persisted for up to 2 consecutive seasons, after an administration protocol that included 6 injections only [129].

Another ragweed pollen AIT approach used an ultra-short treatment protocol in order to administer an allergoid coupled with a TLR4 agonist, monophosphoryl lipid A, which acts as an adjuvant. This product has been shown to be safe and it induced a significant improvement in the symptom score [100]. Until now only a few clinical trials with ragweed AIT have been performed in Europe. The most recent one investigated the efficacy of SLIT with Amb a 1 as a tablet, while the others were designed for evaluation of the efficacy and safety of ragweed allergoids or extracts, either for SCIT or for SLIT (Table 3) [130].

The INSPIRED research project (<http://inspired.onco-gen.ro/en/at-a-glance/>) has been launched in Timisoara, Romania, in an attempt to characterize all ragweed allergens in more detail by using recombinant ragweed allergens, especially regarding their clinical relevance, which is information that is still missing and urgently needed. The results from the identification of clinically important allergens and expression of these recombinant allergens will be used for the development of serological tests, which should be more accurate than the current tests. Based on these results, the project also intends to develop quantitative tests for environmental measurement of ragweed allergen load in order to develop preventive measures (Fig. 5). Another aim of the INSPIRED project is the identification of IgE epitopes of the ragweed pollen allergens. This knowledge can be the basis for novel and better forms of ragweed pollen AIT, a concept comparable with the BM32 for grass pollen allergy [131–134].

Costs of Ragweed Allergy Therapy

As has been shown for all types of immunotherapeutics, the costs of ragweed AIT can be substantial. When it comes to the two main types of therapeutics – symptom-

Table 3. Current ragweed AIT clinical trials in Europe included in EudraCT

EudraCT	Initiation date	Full title of the trial	Product name and code	Main objective of the trial	Primary endpoint	Controlled and randomized	Double-blind	Placebo	Sites, Patients, n
2014-004341-27	Sep. 22, 2015	A phase III, randomized, placebo-controlled clinical trial to study the efficacy and safety of MK-3641, a ragweed (<i>A. artemisiifolia</i>) sublingual immunotherapy tablet, in children with a history of ragweed-induced rhinoconjunctivitis with or without asthma	Ragweed (<i>A. artemisiifolia</i>) sublingual immunotherapy tablet (MK-3641)	To evaluate the efficacy of the MK-3641 sublingual immunotherapy tablet (12 Amb a 1-U) vs. placebo in the treatment of children 5–17 years of age with ragweed pollen-induced rhinoconjunctivitis, with or without asthma, based on the TCS (sum of rhinoconjunctivitis DSS and rhinoconjunctivitis DMS) averaged over the peak RS	The primary endpoint of this study, the TCS, is a combination of (sum of) the rhinoconjunctivitis DSS and the rhinoconjunctivitis DMS averaged over the peak ragweed pollen season	Yes	Yes	Yes	5 1,000
2014-004431-38	Jan. 7, 2015	Double-blind, placebo-controlled, parallel-group, randomized multicenter study to assess the efficacy and safety of the LAIS® ragweed sublingual tablet in patients with allergic rhinoconjunctivitis to ragweed pollen	Lais® ragweed maintenance therapy	To evaluate the efficacy of a 4- to 5-month SLIT treatment with a ragweed monomeric allergoid (Lais ragweed) administered at 1,000 UA/day in ragweed-allergic patients with rhinoconjunctivitis with/without concomitant asthma, due to ragweed pollen by means of the TCS	To assess the efficacy of sublingual immunotherapy with the allergoid LAIS® ragweed sublingual tablets (between the visits V2 and V3, corresponding to the expected ragweed pollen exposition period) The ragweed pollen season is defined as beginning on the first of 3 consecutive recorded days with a pollen count ≥ 10 grains/m ³ and ending on the last day of the last occurrence of 3 consecutive days with a pollen count ≥ 10 grains/m ³ The analysis will be focused on the pollen peak defined as the 15 consecutive recorded days within the RS with the highest 15-day moving average pollen count for each site Pollen regions will be defined according to pollen stations and included sites within an acceptable distance from pollen counters (counters were within approx. 50 miles from the subject's home)	Yes	Yes	Yes	19 228
2011-004522-10	Mar. 2, 2012	Study on the efficacy and safety of 3 different doses of Lais Ambrosia tablets in patients with allergic rhinoconjunctivitis to pollen of <i>Ambrosia</i>	Lais Ambrosia tablets 300, 1,000, and 2,000 UA	To assess the efficacy and safety of treatment with sublingual specific immunotherapy (antiallergic vaccination) with allergoid monomeric ragweed (Lais Ambrosia) in 3 different doses in patients with rhinoconjunctivitis to ragweed pollen	The efficacy of immunotherapy with sublingual allergoid Lais Ambrosia tablets will be assessed through the measurement of individual variation in specific nasal provocation tests performed with <i>Ambrosia</i> allergen	Yes	Yes	No, same IMP used at different dosages	na na

Table 3 (continued)

EudraCT	Initiation date	Full title of the trial	Product name and code	Main objective of the trial	Primary endpoint	Controlled and randomized	Double-blind	Placebo	Sites, Patients, n
2011-001682-41	Dec. 6, 2011	Comparison of the efficacy and the safety of different schedules of administration of sub-lingual immunotherapy in patients with ragweed pollinosis: a phase III randomized and controlled clinical study	Ragweed pollen extract	To evaluate the percentage of CD14–PDL-1–IL10+ circulating allergen-specific ragweed pollen-allergic patients undergoing a preseasonal regimen of SLIT administered sublingually vs. oral vestibular To assess the percentage of CD14–PDL-1–IL10+ circulating allergen-specific ragweed pollen-allergic patients undergoing a preseasonal regimen of SLIT administered sublingually at a dose doubled to 400 STU/dose vaccine compared to the commonly used marketing 200 STU/dose	The end of the study will detect a difference between groups with respect to the primary objective, the percentage of CD14–PDL-1–IL10+ outstanding of at least 25 percentile points	Yes	No, open	Yes	1 na
2008-003864-20	Nov. 19, 2008	A multicenter, double-blind, randomized, placebo-controlled, parallel-group study evaluating the efficacy and long-term safety of a ragweed (<i>A. artemisiifolia</i>) sublingual tablet (SCH 39641) in adult subjects with a history of ragweed pollen-induced rhinoconjunctivitis with or without asthma	SCH 039641 SL tablet SCH 039641 1.5, 6, 12 Amb a 1 units	To evaluate the efficacy of the ragweed sublingual tablet (SCH 39641) vs. placebo in the treatment of ragweed pollen-induced rhinoconjunctivitis based on combined (sum of) rhinoconjunctivitis DSS and rhinoconjunctivitis DMS averaged over the entire RS	The primary efficacy endpoint is the combined (sum of) rhinoconjunctivitis DSS and DMS averaged over the entire RS The combined score for each subject will be calculated as the sum of rhinoconjunctivitis DSS and DMS during the entire RS, divided by the duration of the entire RS	Yes	Yes	Yes	22 na
2006-005713-35	Mar. 9, 2007	A randomized, double-blind, parallel group, placebo-controlled study to assess the efficacy and safety of oral microencapsulated ragweed pollen extract administered prior to and during the ragweed pollen season	Microencapsulated ragweed pollen extract (MRPE)	To evaluate the efficacy and safety of an orally administered microencapsulated ragweed pollen extract, 40 Amb a 1 units daily, and placebo initiated at least 8 weeks prior to, and continuing throughout, the ragweed pollen season in ragweed pollen-allergic subjects The primary efficacy measure will be the average daily total symptom score of 7 symptoms during the peak ragweed pollen period	The primary efficacy measure will be the average total symptom score during the 3 contiguous peak ragweed pollen weeks or the ragweed pollen season if the peak season is less than 3 weeks The total symptom score will include the following 7 symptoms: 1, nasal stuffiness/congestion; 2, nasal discharge/postnasal drip; 3, nasal itching; 4, sneezing; 5, itchy/burning eyes; 6, tearing/watering eyes; 7, itchy throat and/or ears	Yes	Yes	Yes	5 na

TCS, total combined score; DSS, daily symptom score; DMS, daily medication score; RS, ragweed season; UA, units of allergy; STU, standard therapeutic unit; IMP, investigational medicinal product; na, information not available.

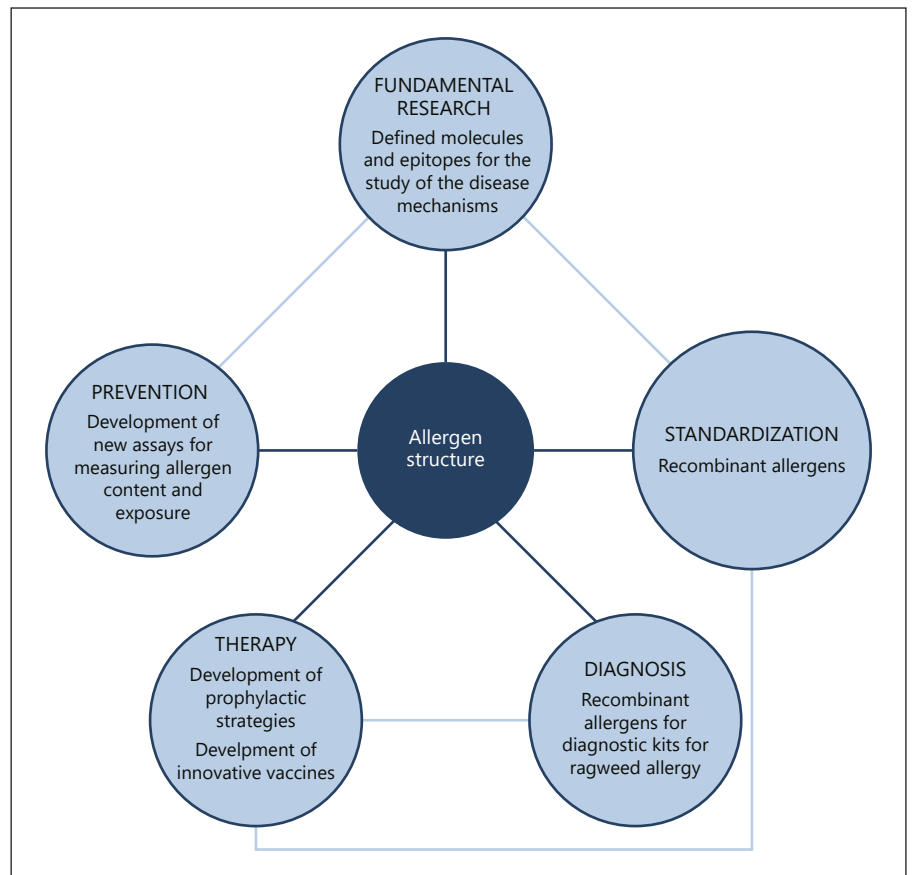


Fig. 5. Overview of the INSPIRED project. The project aim is to characterize all ragweed allergens in more detail which will have an effect on allergy prevention, fundamental research, allergy diagnosis, and finally allergy therapy.

atic or immunotherapy – symptomatic treatment may be cheaper per dose, but it is usually required every day during the time of allergen exposure, whereas AIT can be more expensive in the short-term, but it is not required daily and, moreover, after completing the treatment it provides long-term protection.

Besides the medication costs, there are other expenses associated with ragweed pollen allergy, such as doctor's visits, admittance into hospital, sick leave, or devices for limiting pollen exposure. A European survey provided data regarding the considerable treatment costs for ragweed pollen allergy in different countries (Table 4) [135].

Medication costs may change in the future as new, more effective drugs are created and introduced onto the market, or as patents run out. Cost evolution is difficult to predict because the innovative drugs released on the market may be expensive, but on the other hand the increased effectiveness may reduce future health costs of ragweed pollen-induced allergy.

When establishing cost-effectiveness, several aspects should be taken into consideration, including the efficacy

Table 4. Treatment costs for ragweed allergy in Europe [135]

Country	Cost per patient, EUR	Total cost per year, EUR
Austria	630	88 million
Czech Republic	8.3 (antihistamines) 43.5 (AIT)	Not available
Germany	650	17–47 million
Hungary	Not available	100 million
Switzerland	24 (antihistamines) 484–645 (AIT)	8–24 million
Italy	Not available	1.74 million
Serbia	547–2,555	Not available
France	26–386 reimbursement	Not available
UK	3,030 over 10 years	Not available

of symptom relief during the first years of therapy, sustained efficacy and disease modification treatment outcomes, and sustained lack of symptoms after discontinuation of therapy. The practitioner has to balance between

the level of evidence and the risk-benefit ratio when choosing a certain AIT product, as the primary concerns should be the patient's quality of life and long-term effects of the drug [87].

Conclusion

A. artemisiifolia, also known as common or short ragweed, is an invasive and noxious herbaceous plant that exists worldwide. Climate change and urbanization are key factors behind an accelerated spread of ragweed, which in turn will also increase the ragweed-related burden in many areas. Ragweed pollen allergy will become a serious healthcare problem exerting a strong negative impact on the quality of life of allergic patients. Currently, 11 ragweed allergens have been described and characterized, but in many cases information about their clinical relevance is missing. Therefore, precise diagnosis and effective treatment are not yet established for ragweed pollen allergy. There are therapeutic options for symptomatic treatment, but in many patients they cannot achieve total symptom control.

Currently, diagnosis as well as AIT, which represents the only treatment that can alter the natural course of the allergic disease, are mainly based on ragweed pollen extracts and they have their limits regarding precision in diagnosis and efficacy in treatment. Innovative strategies for ragweed AIT are under development but no promising candidates are present. Therefore, more efforts have to be invested in this field. Thus the INSPIRED research project was launched at the Center for Gene- and Cellular Therapies in the Treatment of Cancer (OncoGen, www.oncogen.ro) in Timisoara, Romania, in order to produce recombinant ragweed allergens for the component resolved diagnosis and also to find new and innovative treatment strategies.

Disclosure Statement

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online suppl. Table S1a. Amb a 1 Homology

Protein	NCBI Acc. #	Amb a 1.01	Amb a 1.04	Amb a 1.03	Amb a 1.02	Amb a 1.05	pectate lyase 1	Art v 6	Cha o 1	Cry j 1	Jun v 1	Cup s 1	Jun a 1	Jun v 1	Cup a 1
Amb a 1.01	P27759.1	100													
Amb a 1.04	P28744.1	82.05	100												
Amb a 1.03	P27761.1	76.26	72.12	100											
Amb a 1.02	P27760.1	76.52	74.42	86.9	100										
Amb a 1.05	P27762.1	67.59	63.33	70.96	70.78	100									
pectate lyase 1 [<i>Helianthus annuus</i>]	OTF85893.1	67.86	65.12	74.55	73.86	69.21	100								
Art v 6 [<i>Art. vulgaris</i>]	AAX85388.1	58.38	57.33	63.29	63.13	59.24	62.76	100							
Cha o 1 [<i>Chamaecyparis obtusa</i>]	Q96385.1	45.14	45.21	47.57	45.95	46.22	47.57	46.49	100						
Cry j 1 [<i>Cryptomeria japonica</i>]	P18632.3	45.41	44.66	45.14	44.59	47.03	47.03	46.22	78.88	100					
Jun v 1 [<i>Juniperus virginiana</i>]	Q9LLT1.1	45.18	45.25	47.38	46.01	46.01	46.56	46.56	85.01	78.47	100				
Cup s 1 [<i>Cupressus sempervirens</i>]	AAF72626.1	44.9	44.41	46.56	45.18	45.73	46.01	45.73	83.92	77.93	96.46	100			
Jun a 1 [<i>Juniperus ashei</i>]	P81294.1	45.18	44.97	47.38	46.01	46.28	46.83	46.28	85.56	78.75	96.46	96.19	100		
Jun v 1 [<i>Juniperus virginiana</i>]	AAF80164.1	44.9	44.97	47.11	45.73	45.73	46.28	46.28	84.74	78.2	99.73	96.19	96.19	100	
Cup a 1 [<i>Cupressus arizonica</i>]	Q9SCG9.2	44.08	43.85	46.01	44.63	45.18	45.18	46.28	83.92	78.2	94.28	95.64	94.82	94.01	100

Values in the table represents percent homology

Percent homology was determined by performing first a NCBI blast followed by Clustal Omega alignment calculating the percentage homology

online suppl. Table S1b. Amb a 3 Homology

Protein	NCBI Acc. #	Amb a 3	Amb a 3-like protein	Plastocyanin-like protein	Amb a 3-like protein
Amb a 3	P00304.2	100			
Amb a 3-like protein [<i>Helianthus annuus</i>]	OTG03928.1	63.16	100		
Plastocyanin-like protein [<i>Corchorus capsularis</i>]	OMO81115.1	46.75	40.66	100	
Amb a 3-like protein [<i>Phoenix dactylifera</i>]	XP_008811152.1	29.73	28.41	25	100

online suppl. Table S1c. Amb a 4 homology

Protein	NCBI Acc. #	Amb a 4	Par h I	Defensin-like protein	Art an 1.0101	Art si 1.0102	Art v 1-like protein	Art gm 1.0102	Art v 1-like protein	Art ar 1.0102	Art v 1
Amb a 4	CBK52317.1	100									
Par h I [<i>Parthenium hysterophorus</i>]	AKF12278.1	71.32	100								
Defensin-like protein [<i>Helianthus annuus</i>]	P22357.1	61.74	81.82	100							
Art an 1.0101 [<i>Artemisia annua</i>]	ANC85006.1	53.91	51.69	52.10	100						
Art si 1.0102 [<i>Artemisia sieversiana</i>]	ANC85016.1	53.91	51.69	52.10	99.24	100					
Art v 1-like protein [<i>Artemisia annua</i>]	AHF71022.1	52.53	53.19	52.34	99.07	98.15	100				
Art gm 1.0102 [<i>Artemisia gmelinii</i>]	ANC85012.1	52.17	50.00	50.42	96.21	96.97	95.37	100			
Art v 1-like protein [<i>Artemisia absinthium</i>]	AHF71021.1	51.52	53.19	53.27	97.22	98.15	96.30	95.37	100		
Art ar 1.0102 [<i>Artemisia argyi</i>]	ANC85009.1	51.30	50.00	50.42	96.97	97.73	96.30	96.97	98.15	100	
Art v 1 [<i>Artemisia vulgaris</i>]	Q84ZX5.1	50.43	50.00	50.42	94.70	95.45	95.37	96.21	95.37	96.21	100

online suppl. Table S1d . Amb a 5 homology

Protein	NCBI Acc. #	Amb a 5	Amb p 5a	Amb p 5b	Amb t5
Amb a 5	P02878.1	100			
Amb p 5a [<i>Ambrosia psilostachya</i>]	P43174.1	95.56	100		
Amb p 5b [<i>Ambrosia psilostachya</i>]	P43175.1	66.67	77.92	100	
Amb t 5 [<i>Ambrosia trifida</i>]	P10414.2	45.00	43.55	45.16	100

online suppl. Table S1e. Amb a 6 homology

Protein	NCBI Acc. #	Amb a 6	nsLTP [Hel. annuus]	Pla o 3	Pru d 3	Mor n 3	Pru ar 3	Pru p 1	Pru p 3	Sin a 3	Api g 2	Act d 10.01	Ara h 9	Art an 3.0101	Rub i 3	Par j 1	Art gm 3.0101	Pru av 3	Pru du 3	Mal d 3	Zea m 14	Cor a 8	Art v 3	Pyr c 3	Art la 3.0101	Art ar 3.0102	Par j 2.0102
Amb a 6	O04004.1	100																									
nsLTP [<i>Helianthus annuus</i>]	OTF92638.1	51.72	100																								
Pla o 3 [<i>Platanus orientalis</i>]	ABY21307.1	38.53	30.28	100																							
Pru d 3 [<i>Prunus domestica</i>]	P82534.1	38.20	33.71	59.34	100																						
Mor n 3 [<i>Morus nigra</i>]	P85894.1	38.20	32.58	63.74	70.33	100																					
Pru ar 3 [<i>Prunus armeniaca</i>]	P81651.2	37.08	33.71	61.54	92.31	68.13	100																				
Pru p 1 [<i>Prunus persica</i>]	P81402.1	35.96	31.46	60.44	90.11	68.13	91.21	100																			
Pru p 3 [<i>Prunus persica</i>]	2ALG_A	35.56	31.11	59.78	90.11	70.33	92.31	97.80	100																		
Sin a 3 [<i>Sinapis alba</i>]	ABU95411.1	35.56	35.56	46.74	53.85	54.95	54.95	54.95	54.35	100																	
Api g 2 [<i>Apium graveolens</i>]	E6Y8S8.1	33.94	29.36	49.56	54.95	53.85	54.95	53.85	53.26	45.65	100																
Act d 10.01 [<i>Actinidia deliciosa</i>]	P86137.2	33.71	30.34	51.65	42.22	47.78	42.22	46.67	45.05	41.76	48.35	100															
Ara h 9 [<i>Arachis hypogaea</i>]	ABX75045.1	33.71	30.34	51.65	60.44	64.84	59.34	61.54	61.54	56.04	54.95	55.56	100														
Art an 3.0101 [<i>Artemisia annua</i>]	ANC85017.1	33.33	28.70	51.75	49.45	57.14	49.45	50.55	50.00	46.74	46.09	41.76	52.75	100													
Rub i 3 [<i>Rubus idaeus</i>]	ABG54494.1	33.33	29.63	53.85	74.73	72.53	68.13	68.13	68.48	53.26	50.89	43.96	62.64	45.13	100												
Par j 1 [<i>Parietaria judaica</i>]	P43217.3	32.97	34.07	33.70	26.37	29.67	28.57	29.67	28.26	28.26	32.61	30.77	28.26	29.35	27.17	100											
Art gm 3.0101 [<i>Artemisia gmelinii</i>]	ANC85022.1	32.41	27.78	51.75	50.55	58.24	52.75	51.65	51.09	48.91	45.22	40.66	51.65	95.73	46.90	30.43	100										
Pru av 3 [<i>Prunus avium</i>]	Q9M5X8.1	32.41	28.70	55.56	87.91	70.33	85.71	87.91	86.96	56.52	50.00	43.96	61.54	47.79	70.94	27.17	50.44	100									
Pru du 3 [<i>Prunus dulcis</i>]	AGR27933.1	32.39	30.00	47.37	58.33	56.67	51.67	53.33	52.46	47.54	35.53	52.46	50.00	39.47	50.67	26.23	39.47	50.67	100								
Mal d 3 [<i>Malus domestica</i>]	AAR22488.1	32.08	30.19	56.52	83.52	70.33	86.81	80.22	80.43	52.17	49.09	41.76	62.64	44.14	74.78	27.17	46.85	82.61	50.68	100							
Zea m 14 [<i>Zea mays</i>]	P19656.1	31.53	29.73	48.28	58.24	64.84	59.34	61.54	60.87	53.26	46.02	49.45	61.54	41.74	53.91	30.11	42.61	50.43	43.24	53.10	100						
Cor a 8 [<i>Corylus avellana</i>]	4XUW_A	31.11	27.78	51.09	61.54	62.64	60.44	59.34	58.70	51.09	50.00	41.76	54.95	52.17	60.87	23.91	53.26	58.70	52.46	60.87	50.00	100					
Art v 3 [<i>Artemisia vulgaris</i>]	ACE07187.1	30.84	26.85	53.98	51.65	61.54	52.75	51.65	51.09	51.09	45.61	43.96	50.55	87.93	49.11	30.43	91.38	50.00	42.67	46.36	42.98	56.52	100				
Pyr c 3 [<i>Pyrus communis</i>]	Q9M5X6.1	30.19	30.19	59.13	79.12	64.84	78.02	78.02	77.17	48.91	49.09	40.66	60.44	49.55	70.43	29.35	49.55	79.13	47.95	87.83	51.33	55.43	50.91	100			
Art la 3.0101 [<i>Artemisia lavandulifolia</i>]	ANC85024.1	29.63	25.00	50.00	46.15	56.04	47.25	46.15	45.65	44.57	43.48	43.96	47.25	85.47	45.13	29.35	88.03	46.90	38.16	43.24	39.13	51.09	87.07	46.85	100		
Art ar 3.0102 [<i>Artemisia argyi</i>]	ANC85020.1	28.97	25.93	51.33	48.35	57.14	50.55	49.45	48.91	51.09	44.74	41.76	48.35	89.66	45.54	28.26	92.24	48.21	40.00	44.55	41.23	53.26	93.97	48.18	86.21	100	
Par j 2.0102 [<i>Parietaria judaica</i>]	O04403.1	27.93	27.27	32.73	31.11	31.11	31.11	27.78	27.78	26.67	28.83	32.58	29.67	29.63	29.36	48.51	31.48	26.61	28.00	26.17	32.73	28.89	30.84	25.23	30.56	29.91	100

online suppl. Table S1f. Amb a 8 homology

Protein	NCBI Acc. #	Amb a 8.0101	Profilin [<i>H. annuus</i>]	Amb a 8.0102	Art v 4.01	Hel a 2	Phl p 11	Bet v 2	Cor a 2	Ole e 2	Phl p 12	Nic t 8	Mera 1	Ama r 2	Hev b 8.0201	Fra e 2.01	Pyr c 4	Zea m 12	Par j 3.0201	Che a 2	Lit c 1	Mal d 4.0101	Sin a 4	Sal k 4.03	Api g 4	Cyn d 12	Ama v 1.01	Dau c 4	Gly m 3.0101	Mus a 1	Cap a 2	Pru du 4.1	Ara h 5	Fra a 4	Pru av 4	Cro s 1	Cit s 2	Par j 3.0101	Pru p 4.02		
Amb a 8.0101	AAX77687.1	100																																							
Profilin [<i>Helianthus annuus</i>]	OTG03788.1	93.98	100																																						
Amb a 8.0102	AAX77688.1	91.73	92.48	100																																					
Art v 4.01 [<i>Artemisia vulgaris</i>]	Q8H2C9.3	89.47	87.97	87.97	100																																				
Hel a 2 [<i>Helianthus annuus</i>]	O81982.1	81.20	83.46	81.20	84.21	100																																			
Phl p 11 [<i>Phleum pratense</i>]	P35079.1	80.15	80.92	78.63	77.86	83.21	100																																		
Bet v 2 [<i>Betula verrucosa</i>]	A4K9Z8.1	79.70	78.95	76.69	77.44	72.18	80.15	100																																	
Cor a 2 [<i>Corylus avellana</i>]	A4KA45.1	78.95	78.95	76.69	76.69	72.93	80.92	96.99	100																																
Ole e 2 [<i>Olea europaea</i>]	A4GFB7.1	78.95	78.20	76.69	77.44	75.94	80.92	84.21	83.46	100																															
Phl p 12 [<i>Phleum pratense</i>]	A4KA33.1	78.63	79.39	77.10	77.86	82.44	96.18	80.92	81.68	81.68	100																														
Nic t 8 [<i>Nicotiana tabacum</i>]	Q9ST99.1	78.20	77.44	75.94	75.94	73.68	80.92	82.71	83.46	82.84	80.15	100																													
Mer a 1 [<i>Mercurialis annua</i>]	Q49894.1	77.44	77.44	75.94	75.94	75.94	83.21	87.97	90.98	81.95	81.68	81.95	100																												
Ama r 2 [<i>Amaranthus retroflexus</i>]	ACP43298.1	75.94	75.94	74.44	77.94	78.95	80.15	75.94	76.69	81.20	79.39	76.69	76.69	100																											
Hev b 8.0201 [<i>Hevea brasiliensis</i>]	Q9M7N0.1	76.34	75.57	76.34	76.34	77.10	80.92	83.21	84.73	80.92	80.15	79.39	82.44	83.21	100																										
Fra e 2.01 [<i>Fraxinus excelsior</i>]	AHL24660.1	75.94	75.19	74.44	78.20	73.68	79.39	87.22	87.97	88.06	80.15	80.60	84.21	80.45	81.68	100																									
Pyr c 4 [<i>Pyrus communis</i>]	Q9XF38.1	74.81	73.28	74.81	74.81	70.99	77.10	84.73	86.26	82.44	77.86	83.21	82.44	82.44	87.79	87.02	100																								
Zea m 12 [<i>Zea mays</i>]	P35081.1	74.81	72.52	74.81	75.57	76.34	82.44	77.10	78.63	78.63	81.68	77.86	78.63	79.39	87.79	78.63	78.63	100																							
Par j 3.0201 [<i>Parietaria judaica</i>]	CCP19647.1	74.05	74.05	73.28	78.63	74.81	80.15	79.39	78.63	80.15	79.39	77.86	77.86	85.50	83.21	80.92	82.44	79.39	100																						
Che a 2 [<i>Chenopodium album</i>]	Q84V37.1	74.81	74.05	73.28	75.57	74.05	80.92	76.34	78.63	77.86	77.86	80.92	78.63	77.10	83.97	77.86	79.39	80.15	79.39	100																					
Lit c 1 [<i>Litchi chinensis</i>]	Q941H7.1	74.81	74.05	74.05	76.34	73.28	80.15	81.68	82.44	79.39	80.15	80.15	83.21	88.55	81.68	84.73	83.21	82.44	82.44	100																					
Mal d 4.0101 [<i>Malus domestica</i>]	Q9XF42.1	73.28	73.28	74.81	75.57	75.57	76.34	79.39	80.92	81.68	76.34	80.15	80.15	83.21	83.21	82.44	83.21	78.63	83.97	77.86	82.44	100																			
Sin a 4 [<i>Sinapis alba</i>]	ABU95412.1	73.28	73.28	71.76	74.05	75.57	77.86	77.86	80.15	81.68	77.86	77.86	80.92	79.39	75.57	83.21	79.39	72.52	81.68	78.63	79.39	81.68	100																		
Sal k 4.03 [<i>Salsola kali</i>]	AHL24658.1	72.93	72.93	72.93	77.44	76.69	74.81	75.19	75.94	78.20	74.05	74.44	74.44	87.97	80.15	78.95	80.92	75.57	83.97	76.34	82.44	80.15	76.34	100																	
Api g 4 [<i>Apium graveolens</i>]	Q9XF37.1	72.93	73.68	69.92	73.68	72.18	77.86	81.95	83.46	82.09	78.63	78.36	80.45	78.95	80.92	82.84	83.97	77.10	80.92	74.05	81.68	79.39	80.15	75.94	100																
Cyn d 12 [<i>Cynodon dactylon</i>]	O04725.1	72.52	72.52	71.76	75.57	76.34	82.44	74.81	76.34	77.86	81.68	77.86	77.86	81.68	83.97	80.15	81.68	86.26	82.44	80.15	82.44	80.92	76.34	76.34	80.15	100															
Ama v 1.01 [<i>Amaranthus viridis</i>]	ABW37744.1	72.18	72.18	74.44	72.93	70.68	74.05	77.44	76.69	77.44	74.05	73.68	76.69	86.47	82.44	78.20	80.15	75.57	84.73	77.10	83.97	85.50	77.10	84.21	78.20	78.63	100														
Dau c 4 [<i>Daucus carota</i>]	Q8SAE6.1	72.18	72.18	71.43	72.18	69.92	77.86	83.46	84.96	79.85	77.86	78.36	82.71	76.69	81.68	79.85	84.73	77.86	78.63	74.81	80.92	79.39	78.63	75.19	91.04	78.63	78.95	100													
Gly m 3.0101 [<i>Glycine max</i>]	Q65809.1	71.76	70.23	69.47	74.05	71.76	76.34	76.34	77.10	79.39	76.34	80.15	76.34	82.44	82.44	80.92	82.44	79.39	80.92	82.44	83.97	79.39	78.63	77.86	78.63	80.92	79.39	76.34	100												
Mus a 1 [<i>Musa acuminata</i>]	Q94JN3.1	71.76	70.99	70.99	72.52	71.76	74.81	80.15	82.44	77.86	74.81	79.39	80.92	77.10	83.97	81.68	83.97	80.15	77.86	81.68	85.50	81.68	77.10	74.05	81.68	80.15	78.63	78.63	84.73	100											
Cap a 2 [<i>Capsicum annuum</i>]	Q93YI9.1	70.99	70.23	69.47	74.81	72.52	78.63	77.86	78.63	77.10	78.63	78.63	77.86	83.97	83.21	82.44	81.68	80.92	86.26	80.92	85.50	82.44	82.44	83.21	79.39	80.92	86.26	80.15	86.26	79.39	100										
Pru du 4.1 [<i>Prunus dulcis</i>]	AGR27934.1	70.73	71.95	73.17	75.61	71.95	71.95	75.61	78.05	76.83	71.95	81.71	75.61	82.93	81.71	82.93	85.37	75.61	86.59	75.61	81.71	97.56	81.71	79.27	76.83	79.27	82.93	76.83	79.27	81.71	85.37	100									
Ara h 5 [<i>Arachis hypogaea</i>]	Q95QI9.1	70.23	68.70	67.94	69.47	67.94	75.57	74.05	74.81	73.28	76.34	74.81	71.76	75.57	77.86	74.05	78.63	75.57	79.39	80.15	79.39	74.05	75.57	73.28	75.57	74.81	76.34	76.34	83.21	78.63	84.73	71.95	100								
Fra a 4 [<i>Fragaria x ananassa</i>]	POCOY3.1	69.47	70.23	69.47	70.23	71.76	78.63	74.81	77.86	77.86	77.86	77.86	78.63	82.44	82.44	78.63	80.15	77.86	84.73	82.44	84.73	80.15	82.44	80.15	80.15	83.97	81.68	83.21	79.39	87.79	80.49	80.15	100								
Pru av 4 [<i>Prunus avium</i>]	Q9XF39.1	69.47	69.47	70.99	73.28	73.28	74.05	77.10	78.63	78.63	74.05	79.39	77.10	83.21	84.73	82.44	85.50	77.86	85.50	76.34	82.44	93.13	79.39	82.44	77.86	80.1															

online suppl. Table S1g. Amb a 9 homology

Protein	NCBI Acc. #	Amb a 9	Nic t 1-like CBP	Art v 5	Syr v 3	Bet v 4	Ole e 3	Bra n 2	Che a 3	Aln g 4	Phl p 7	Cyn d 7	Jun o 2	CBP	Ole e 8
Amb a 9 [<i>Ambrosia artemisiifolia</i>]	AAX77684.1	100													
Nic t 1-like CBP [<i>Helianthus annuus</i>]	OTF94741.1	91.57	100												
Art v 5 [<i>Artemisia vulgaris</i>]	AAX85389.1	80.49	80.49	100											
Syr v 3 [<i>Syringa vulgaris</i>]	P58171.1	70.37	65.43	64.2	100										
Bet v 4 [<i>Betula verrucosa</i>]	Q39419.1	68.67	65.06	65.85	76.54	100									
Ole e 3 [<i>Olea europaea</i>]	O81092.1	68.67	63.86	63.41	90.12	78.57	100								
Bra n 2 [<i>Brassica napus</i>]	P69198.1	67.47	65.06	64.63	74.07	72.29	73.49	100							
Che a 3 [<i>Chenopodium album</i>]	Q84V36.1	66.27	66.27	62.20	77.78	81.18	80.95	69.88	100						
Aln g 4 [<i>Alnus glutinosa</i>]	O81701.1	65.06	62.65	63.41	76.54	89.41	82.14	75.90	83.53	100					
Phl p 7 [<i>Phleum pratense</i>]	O82040.1	58.97	61.54	58.97	67.95	67.95	67.95	64.10	73.08	69.23	100				
Cyn d 7 [<i>Cynodon dactylon</i>]	P94092.2	57.50	61.25	60.00	63.75	67.50	63.75	60.00	71.25	66.25	92.31	100			
Jun o 2 [<i>Juniperus oxycedrus</i>]	O64943.2	46.05	43.42	44.00	51.35	48.72	46.75	50.00	45.57	50.00	49.30	46.58	100		
CBP [<i>Olea europaea</i>]	AAF31152.1	44.58	42.17	42.68	51.85	48.24	51.19	48.19	45.35	47.06	46.15	45.00	40.88	100	
Ole e 8 [<i>Olea europaea</i>]	Q9M7R0.1	43.37	40.96	41.46	50.62	45.88	50.00	45.78	43.02	44.71	43.59	42.50	40.25	97.66	100

online suppl. Table S1h. Amb a 10 homology

Protein	NCBI Acc. #	Amb a 10	EF-hand domain pair	Ole e 8	Bet v 4	Cyn d 7	Che a 3	Phl p 7	Syr v 3	Aln g 4	Ole e 3	Bra n 2	Jun o 2 / Jun o 4	CBP	Bla g 6.0101	Bet v 3
Amb a 10	AAX77686.1	100														
EF-hand domain pair [<i>H. annuus</i>]	OTF93129.1	87.50	100													
Ole e 8 [<i>Olea europaea</i>]	Q9M7R0.1	53.75	52.50	100												
Bet v 4 [<i>Betula verrucosa</i>]	Q39419.1	52.94	49.41	47.06	100											
Cyn d 7 [<i>Cynodon dactylon</i>]	P94092.2	52.50	52.50	43.75	67.50	100										
Che a 3 [<i>Chenopodium album</i>]	Q84V36.1	52.33	48.84	44.19	81.18	71.25	100									
Phl p 7 [<i>Phleum pratense</i>]	O82040.1	51.28	51.28	44.87	67.95	92.31	73.08	100								
Syr v 3 [<i>Syringa vulgaris</i>]	P58171.1	50.62	46.91	51.85	76.54	63.75	77.78	67.95	100							
Aln g 4 [<i>Alnus glutinosa</i>]	O81701.1	50.59	47.06	45.88	89.41	66.25	83.53	69.23	76.54	100						
Ole e 3 [<i>Olea europaea</i>]	O81092.1	48.81	45.24	51.19	78.57	63.75	80.95	67.95	90.12	82.14	100					
Bra n 2 [<i>Brassica napus</i>]	P69198.1	48.19	46.99	46.99	72.29	60.00	69.88	64.10	74.07	75.90	73.49	100				
Jun o 4 (Jun o 2) [<i>Juniperus oxycedrus</i>]	O64943.2	41.03	40.38	40.25	47.62	45.57	45.88	48.05	50.00	50.00	46.99	48.78	100			
CBP [<i>Arachis hypogaea</i>]	AAZ20284.1	39.35	36.77	38.12	38.82	33.75	36.05	38.46	39.51	38.82	36.90	39.76	47.68	100		
Bla g 6.0101 [<i>Blattella germanica</i>]	ABB89296.1	33.78	35.14	29.80	27.06	27.50	29.41	28.21	30.86	28.24	28.57	26.51	28.47	30.41	100	
Bet v 3 [<i>Betula verrucosa</i>]	P43187.1	30.19	30.19	29.09	27.06	30.00	26.74	30.77	29.63	27.06	27.38	25.30	28.66	30.52	26.49	100

online suppl. Table S1i. Amb a 11 homology

Protein	NCBI Acc. #	Amb a 11	Car p 1	Ale o 1	Act d 1	Act c 1	Ana c 2	Group 1 like	Sui m 1	Aca s 1	Der f 1	Tyr p 1	Blo t 1	Pso o 1	Der p 1	Sar s 1
Amb a 11	AHA56102.1	100														
Car p 1 [<i>Carica papaya</i>]	P00784.1	39.51	100													
Ale o 1 [<i>Aleuroglyphus ovatus</i>]	ABU50816.1	38.94	34.81	100												
Act d 1 [<i>Actinidia deliciosa</i>]	A5HII1.1	36.90	42.60	40.43	100											
Act c 1 [<i>Actinidia chinensis</i>]	P00785.4	36.90	42.60	40.12	98.95	100										
Ana c 2 [<i>Ananas comosus</i>]	O23791.1	34.11	39.44	36.02	41.76	41.47	100									
Group 1 like [<i>Euroglyphus maynei</i>]	OTF70897.1	33.84	32.54	36.78	33.83	33.83	32.42	100								
Sui m 1 [<i>Suidasia medanensis</i>]	AAX34043.1	30.60	30.99	36.56	33.96	33.65	31.21	29.50	100							
Aca s 1 [<i>Acarus siro</i>]	ABU50820.1	29.26	32.14	35.44	33.02	33.02	31.61	27.50	46.63	100						
Der f 1 [<i>Dermatophagoides farinae</i>]	Q58A71	27.27	28.05	30.00	30.29	29.97	28.43	25.00	34.49	34.95	100					
Tyr p 1 [<i>Tyrophagus putrescentiae</i>]	ABM53753.1	26.60	29.13	29.21	31.75	31.75	32.80	29.69	39.81	38.89	31.49	100				
Blo t 1 [<i>Blomia tropicalis</i>]	AAQ24541.1	26.11	30.67	30.94	30.19	29.87	31.53	25.62	40.61	40.68	35.67	38.20	100			
Pso o 1 [<i>Psoroptes ovis</i>]	Q1EIQ3.1	25.24	29.28	30.87	30.84	30.52	30.62	24.60	33.12	35.48	65.73	30.10	33.33	100		
Der p 1 [<i>D. pteronyssinus</i>]	ACG58378.1	23.45	25.83	28.16	29.08	28.76	26.56	22.19	32.06	31.49	82.81	29.64	33.87	62.50	100	
Sar s 1 [<i>Sarcoptes scabiei</i>]	AAS93673.1	23.03	22.64	24.51	24.68	24.68	22.30	23.36	24.50	26.91	26.01	23.67	23.43	26.69	25.42	100