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ORIGINAL ARTICLE

Identification of Amb t 18 as a novel allergen from *Ambrosia trifida* pollen

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Abstract

Background: *Ambrosia* (ragweed) pollen is a major allergenic source, but *Ambrosia trifida* allergens remain understudied. Triosephosphate isomerase, a conserved pan-allergen in plants, has not been reported in weed pollen.

Objective: To identify and characterize the novel allergen (Amb t 18) and evaluated its clinical relevance.

Methods: Amb t 18 cDNA was cloned and expressed in *E. coli*. Natural (nAmb t 18) and recombinant (rAmb t 18) proteins were purified for structural analyses (CD spectra) and IgE-reactivity testing (ELISA/basophil activation). Cross-reactivity with homologs Pla a 7 and Tri a 31 was assessed through inhibition ELISA. Structural analyses included 3D modeling, sequence alignment, and phylogenetics.

Results: Natural and recombinant Amb t 18 exhibited similar CD spectra. Amb t 18 reacted with 35.1% (13/37) of serum samples, inhibited 17.56% of IgE-binding to pollen extracts, and activated basophils. In some sera, its IgE-binding activity exceeded that of ragweed pollen extracts. It shared 82% sequence identity with Pla a 7 and Tri a 31, grouped together in the phylogenetic tree. The recombinant Amb t 18 reacted with Pla a 7 or Tri a 31 IgE-positive sera from *Platanus acerifolia* or *Triticum aestivum*-allergic patients, rAmb t 18, rPla a 7, and rTri a 31 inhibited IgE binding to rAmb t 18 by 76.70%, 5.80%, and 21.94%, respectively.

Conclusions: Amb t 18 was identified as a novel *Ambrosia trifida* pollen allergen, the first of its type in ragweed pollen. These findings may aid in developing new diagnostic and therapeutic approaches for ragweed allergy.

Key words: *Ambrosia trifida*, pollen, allergen, Triosephosphate isomerase, Immunoglobulin E

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Introduction

Ambrosia, a globally invasive species, significantly impacts ecosystems.[1] Ragweed has been recognized as a major health problem in North America. Allergic rhinitis and asthma are the main allergic diseases associated with exposure to ragweed pollen.[2] Some studies have shown that ragweed pollen sensitization rates in the USA are between 15 and 26% of the common population.[3] Among all *Ambrosia* species, *Ambrosia artemisiifolia* (short ragweed) and *Ambrosia trifida* (giant ragweed) are the most common species. Short ragweed, the only deeply investigated species, is a major cause of allergy in late summer worldwide.[4] In contrast, giant ragweed remains understudied despite its expanding global threat.[5] Native to eastern North America, it has invaded Europe and Asia. In China, it is now a government-listed invasive species, with the eastern region identified as a highly suitable habitat.[6] Climate models predict further northward expansion.[7] In Beijing, 12.5% of allergic rhinitis patients show positive skin reactivity to its pollen.[8] Given its non-overlapping distribution with short ragweed[9] and divergent allergenicity,[10] dedicated research on giant ragweed allergens is imperative.

World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee has recorded 12 allergens from short ragweed pollen, however, only 3 allergens (Amb t 5, Amb t 8, Amb t 13) of *Ambrosia trifida* pollen have been identified and officially recorded in the Allergen Nomenclature database (http://www.allergen.org/). Amb t 5 is a 5 kDa allergen with 5% skin reactivity from 200 ragweed-sensitive patients.[11] However, since the discovery of Amb t 5, no new allergens have been discovered and named for giant ragweed in recent decades, and our group has successively discovered and named Amb t 8 and Amb t 13 recently. Compared with the over 10 allergens in short ragweed pollen, many allergens may be still understudied.

Triosephosphate isomerase is an enzyme of the glycolysis pathway which exists in almost all types of cells.[12] Triosephosphate isomerase has been identified as allergens in food,[13] mold[14] and mites.[15] However, the role of triosephosphate isomerase protein family in pollen allergy is rarely studied, our group firstly purified and characterized triosephosphate isomerase as pollen allergen in the tree pollen (Pla a 7 in *Platanus acerifolia*).[16] Whether triosephosphate isomerase was also present in pollen from other sources was a matter of great interest to us. When we screened for potential allergens in giant ragweed pollen, we unexpectedly find this type of protein presented as IgE-binding band in *Ambrosia trifida* pollen extracts, this undoubtedly promoted us to investigate the role of this protein in the weed pollen.

In this study, we matched the theoretical cDNA sequence from transcripts of *Ambrosia trifida* pollen based on mass spectrometry (LC-MS/MS) results of IgE-binding band from extracts. Then, the cDNA sequence was cloned by PCR and further confirmed by Sanger dideoxy sequencing. Both the recombinant (r) Amb t 18 and natural (n) Amb t 18 were purified and the allergenicity was evaluated by IgE-ELISA, inhibition ELISA, Western blot and basophil activation test. This discovery provided a new sight into the role of triosephosphate isomerase as allergens, which is also a further improvement for ragweed pollen allergen information and could be beneficial for the treatment of *Ambrosia* pollen allergy.

Materials and methods

Ragweed pollen and patients' sera

Pollen of giant ragweed was procured from Beijing Macro-Union Pharmaceutical Co. Ltd. (Beijing, China). The commercial pollen crude extracts of short ragweed were purchased from Stallergenes Greer (North Carolina, USA). Ethics approval for this study was granted by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University (2020-SR-401). Patients' sera information details were provided in the supplementary material.

Cloning of the cDNA of rAmb t 18

The theoretical cDNA sequence of Amb t 18 was selected from the transcripts of next-generation sequence of Ambrosia trifida pollen mRNA based on the preliminary results of the identified internal peptides from an approximately 26 kDa IgE-binding band in pollen extracts, the method for cDNA BLAST was the same as we previously described.[17] Total RNAs were extracted using MiniBEST Plant RNA Extraction Kit (Takara, Beijing, China) and reverse-transcribed with cDNA synthesis kit (Takara, Beijing, China). Primers (forward (F), 5'- CAAATCTCATCACAAAGCTT -3'; reverse (R): 5'- TAAAGCCATTGTTGTTCTTC-3') were designed from peptide sequences.

Expression and purification of the rAmb t 18 in E. coli

The rAmb t 18 gene was cloned into pET-28a plasmid using one-step cloning kit (Vazyme, Nanjing, China), inserted between the *Nco* I and *Xho* I restriction sites. The recombinant plasmid was then transferred into *Escherichia coli* BL21 (DE3), and expression of rAmb t 18 was induced using 1 mM isopropyl-β-D-thio-galactopyranoside (IPTG). The recombinant protein was produced with a C-terminal 6 × His-tag. The BL21 (DE3) cells containing rAmb t 18 were lysed by ultrasonication on ice (9s on/5s off, 30 min). The supernatant was purified sequentially with Nickel column (Genscript Biotech Co. Ltd., Nanjing, China) and HiTrap Q HP column (Uppsala, Sweden) using the ÄKTA protein purification system (GE Healthcare, Uppsala, Sweden).

Purification of natural Amb t 18 by immunoaffinity chromatography

The nAmb t 18 was purified by immunoaffinity chromatography from crude extracts using polyclonal antibodies specific for rAmb t 18 (anti-rAmb t 18 pAb). The antibodies were prepared at Zoonbio Biotechnology Co.,Ltd (Nanjing, China). Based on the purification methods already reported by our group.[18] Briefly, the polyclonal antibodies against rAmb t 18 were produced in New Zealand rabbits, which were immunized subcutaneously four times at two-week intervals (1 mg rAmb t 18 per injection) using complete Freund's adjuvant (Sigma-Aldrich, Missouri, USA) for priming and incomplete Freund's adjuvant (Sigma-Aldrich, Missouri, USA) for boosters. Sera were collected 10 days after final immunization. The polyclonal antibodies (rabbit IgG) were purified using a HiTrap rProtein A column according to the manufacturer's instruction (GE Healthcare, Uppsala, Sweden). Then, the purified anti-rAmb t 18 pAb were coupled to cyanogen bromide-activated Sepharose 4B (GE Healthcare, Uppsala, Sweden) as recommended by the manufacturer. The resulting antibody-conjugated resin was then incubated with giant ragweed pollen extracts in PBS containing protease inhibitor cocktail (Beyotime, Shanghai, China) at 4°C overnight. After extensively washed with PBS, then the nAmb t 18 on the gels was eluted with 100 mM glycine-HCl buffer (pH 2.7). The collected fractions were concentrated and analyzed by SDS-PAGE.

Characterization of Amb t 18 and detection of its homolog in short ragweed

The N-terminal amino acid sequences of nAmb t 18 were analyzed by Edman degradation and determined by automated N-terminal sequencing with PPSQ-31A protein sequencer (Shimadzu, Tokyo, Japan) carried out by the APTBIO Co. Ltd (Shanghai, China). The purified nAmb t 18 and rAmb t 18 bands were cut from the gels and digested with trypsin and confirmed by liquid chromatography with tandem LC-MS/MS at Nanjing Positive Function BIOTEC Co., Ltd (Nanjing, China).

The circular dichroism (CD) analysis of nAmb t 18 and rAmb t 18 (in 20 mM sodium phosphate) was carried out on a Chirascan Circular dichroism spectrometer (Applied Photophysics, Leatherhead, UK) in the 200–250 nm wavelength range (Path length: 10 mm, Scan speed: 0.5 seconds per data point, Data pitch: 1 nm, Bandwidth: 1 nm). The K2D3 server was used to calculate the α-helix and β-strand.[19]

Meanwhile, we detected the expression of homologous allergen of Amb t 18 in short ragweed using the anti-rAmb t 18 polyclonal antibodies by Western blot as our previously reported method.[20]

Immunoreactivity of human sera IgE with rAmb t 18

The IgE reactivity of Amb t 18 was evaluated by ELISA using sera from *Ambrosia* pollen-allergic patients, following the previously reported method.[21] The 96-well microplates (Corning Costar, ME, USA) were coated with 1 μg/well rAmb t 18 (4°C overnight). After blocking with 1% BSA for 2 h, patients' sera (1:10 PBST dilution) and HRP-conjugated goat anti-human IgE (KPL, MD, USA) (1:2500 PBST) were sequentially incubated (2 h each). Subsequently, the color was developed using tetramethylbenzidine substrate, and the absorbance was measured at 450 nm. The cut-off value was calculated as the average OD450 of negative controls + 3 standard deviations (SD), the method is widely adopted in allergen research.[22,23]

The giant ragweed pollen extracts, short ragweed pollen extracts and rAmb t 18 were coated on ELISA plates, 5 rAmb t 18-IgE positive patients' sera and 1 serum from a healthy donor were applied to compare their IgE-reactivity, the subsequent procedure was the same as above described. Also, the IgE-reactivity of Amb t 18 with Pla a 7 or Tri a 31-IgE positive sera was investigated by ELISA.

Western blot analysis of Amb t 18

Four representative sera with IgE-absorbance values higher than 1.5-fold cut-off in ELISA were selected for the Western blot. The rAmb t 18 was loaded at 10 μg per lane for detection, whereas nAmb t 18 was subsequently analyzed with loading amount of 2.5 μg per lane due to the limited amount. For both analyses, the purified nAmb t 18 and rAmb t 18 were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Merck Millipore, MA, USA). Blocked membranes were incubated with individual patient sera (1:10 diluted in PBST), followed by HRP-conjugated goat anti-human IgE (KPL, MD, USA) (1:5,000 PBST). IgE-binding bands were visualized using Immobilon™ Western HRP Substrate Luminol Reagent (Merck Millipore) in the Tanon 5200 multi-imaging system (Tanon, Shanghai, China).

Inhibition ELISA test

The method of the inhibition ELISA test was the same as before.[24] The 96-well microplates were coated with 1 μg/well of giant ragweed pollen crude extracts. We selected nine positive sera with a value above 1-fold cut-off in the ELISA experiment to make a positive serum pool (1:10 PBST). Then BSA, rAmb t 18 and crude extracts were divided into 5 concentration gradients (0-10 μg/mL), and were added to the corresponding positive sera and incubated overnight at 4°C, respectively. The sera from healthy donors were used as a control. The next step was the same as the ELISA.

For cross-reactivity, a sera pool (serum samples positive in ELISA) from rAmb t 18-IgE positive patients was pre-incubated with inhibitors (rPla a 7 or rTri a 31 at 10 μg/mL, rAmb t 18 as positive control, BSA as negative control) at 4°C overnight. This pre-incubated mixture was then added to rAmb t 18-coated plates (1 μg/well), the subsequent incubation and detection were the same method as described above.

Basophil Activation Test

The allergenicity of Amb t 18 was assessed by indirect basophil activation test (BAT) as previously described.[18] In BAT, the surface expression of CD63 was recognized as a relevant marker on the surface of activated basophils.[25,26] Basophils were isolated from the blood of healthy donors, and non-specific IgE from the surface of basophils was stripped, the resulted cells were incubated with patient serum to passively sensitize the cells. Then rAmb t 18 (10 μg/mL) was used to stimulate basophils at 37°C for 30 min. Anti-IgE was used as positive control and buffer alone as negative control. The cells were pelleted and mixed with the antibody cocktail consisting of FITC-conjugated anti-CD63 mAb (Biolegend, California, USA) and PE-conjugated anti-CCR3 mAb (Biolegend, CA, USA). The expressions of CD63 and CCR3 on the surface of basophils were evaluated by FACSCanto Plus (BD Bioscience, CA, USA) and analyzed by FlowJo V10 software (OR, USA). The gating threshold was set as 10% $CD63^+$ basophils based on the negative controls according to the previous study.[27]

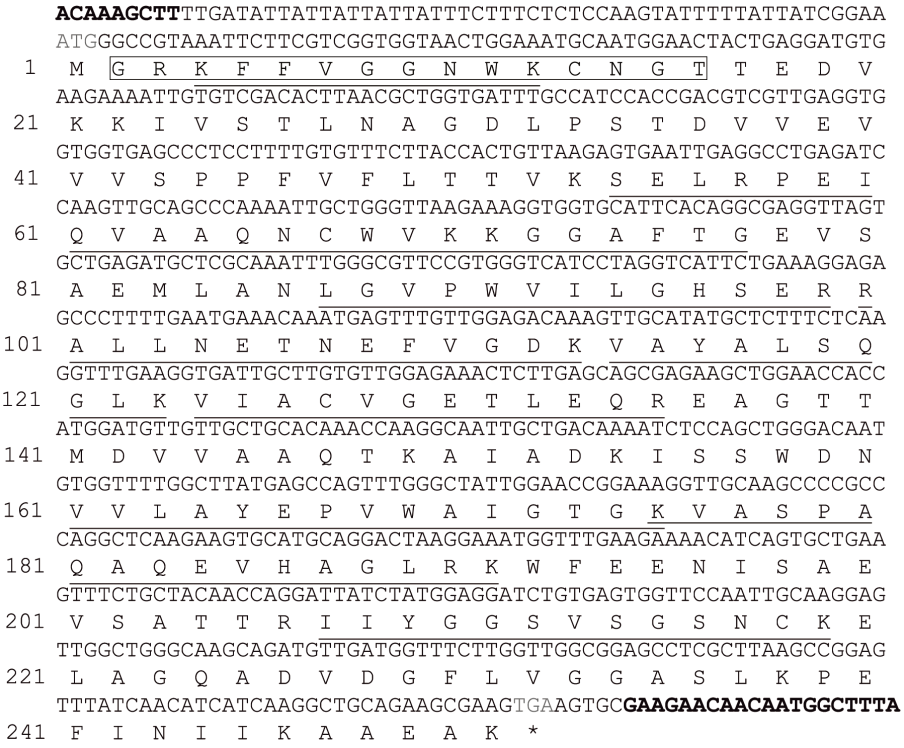
Conservation analysis and structure prediction of Amb t 18

Sequence conservation was analyzed according to the recorded allergens belonging to the triosephosphate isomerase family in WHO/IUIS database. The phylogenetic tree was generated and visualized using MEGA11 software.[28] The sequence identity of triosephosphate isomerase was analyzed by Protein BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and was demonstrated using heat map. Multiple sequences alignment was performed by ClustalX2 software and displayed by ESPript 3.0 (https://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi).[29] The 3-dimensional (3D) structural models were built by SWISS-MODEL (https://swissmodel.expasy.org/) and Root Mean Square Deviation (RMSD) values were calculated by PyMol software (https://pymol.org).[30]

Results

Identification and cloning of Amb t 18

The IgE-Western blot showed that multiple IgE-positive bands could be detected in *Ambrosia trifida* pollen extracts using three different sera pools (each combined from 5 serum) of *Ambrosia* allergic patients. Those bands located at approximately 14 kDa, 26 kDa, 35 kDa to 40 kDa, and a weak band at 70 kDa (**Figure S1**), the triosephosphate isomerase was found in 26 kDa IgE-binding band. The cloned cDNA fragments of Amb t 18 contained a 756 bp open reading frame encoding 251 amino acids (**Figure 1**). The sequence data has been deposited in the GenBank database (Accession No. MW889088).



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     ACAAAGCTTTTGATATTATTATTATTATTTCTTTCTCTCCAAGTATTTTTATTATCGGAA
     ATGGGCCGTAAATTCTTCGTCGGTGGTAACTGGAAATGCAATGGAACTACTGAGGATGTG
  1   M  G  R  K  F  F  V  G  G  N  W  K  C  N  G  T  T  E  D  V
     AAGAAAATTGTGTCGACACTTAACGCTGGTGATTTGCCATCCACCGACGTCGTTGAGGTG
 21   K  K  I  V  S  T  L  N  A  G  D  L  P  S  T  D  V  V  E  V
     GTGGTGAGCCCTCCTTTTGTGTTTCTTACCACTGTTAAGAGTGAATTGAGGCCTGAGATC
 41   V  V  S  P  P  F  V  F  L  T  T  V  K  S  E  L  R  P  E  I
     CAAGTTGCAGCCCAAAATTGCTGGGTTAAGAAAGGTGGTGCATTCACAGGCGAGGTTAGT
 61   Q  V  A  A  Q  N  C  W  V  K  K  G  G  A  F  T  G  E  V  S
     GCTGAGATGCTCGCAAATTTGGGCGTTCCGTGGGTCATCCTAGGTCATTCTGAAAGGAGA
 81   A  E  M  L  A  N  L  G  V  P  W  V  I  L  G  H  S  E  R  R
     GCCCTTTTGAATGAAACAAATGAGTTTGTTGGAGACAAAGTTGCATATGCTCTTTCTCAA
101   A  L  L  N  E  T  N  E  F  V  G  D  K  V  A  Y  A  L  S  Q
     GGTTTGAAGGTGATTGCTTGTGTTGGAGAAACTCTTGAGCAGCGAGAAGCTGGAACCACC
121   G  L  K  V  I  A  C  V  G  E  T  L  E  Q  R  E  A  G  T  T
     ATGGATGTTGTTGCTGCACAAACCAAGGCAATTGCTGACAAAATCTCCAGCTGGGACAAT
141   M  D  V  V  A  A  Q  T  K  A  I  A  D  K  I  S  S  W  D  N
     GTGGTTTTGGCTTATGAGCCAGTTTGGGCTATTGGAACCGGAAAGGTTGCAAGCCCCGCC
161   V  V  L  A  Y  E  P  V  W  A  I  G  T  G  K  V  A  S  P  A
     CAGGCTCAAGAAGTGCATGCAGGACTAAGGAAATGGTTTGAAGAAAACATCAGTGCTGAA
181   Q  A  Q  E  V  H  A  G  L  R  K  W  F  E  E  N  I  S  A  E
     GTTTCTGCTACAACCAGGATTATCTATGGAGGATCTGTGAGTGGTTCCAATTGCAAGGAG
201   V  S  A  T  T  R  I  I  Y  G  G  S  V  S  G  S  N  C  K  E
     TTGGCTGGGCAAGCAGATGTTGATGGTTTCTTGGTTGGCGGAGCCTCGCTTAAGCCGGAG
221   L  A  G  Q  A  D  V  D  G  F  L  V  G  G  A  S  L  K  P  E
     TTTATCAACATCATCAAGGCTGCAGAAGCGAAGTGAAGTGCGAAGAACAACAATGGCTTTA
241   F  I  N  I  I  K  A  A  E  A  K  *
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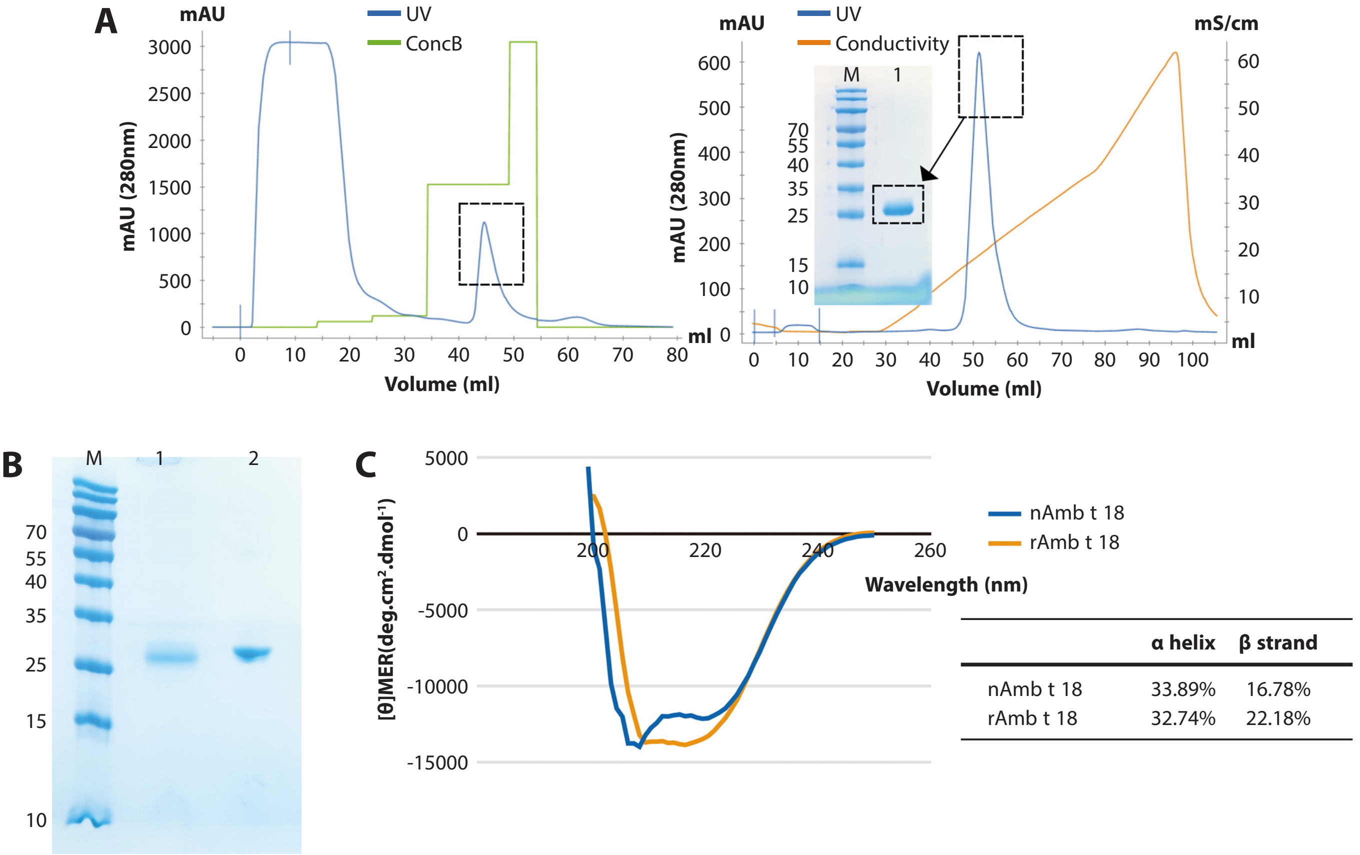
Figure 1. The cloned nucleotide sequence and the deduced amino acid sequence of Amb t 18. The underlined peptides were the identified internal peptides of nAmb t 18 by LC-MS/MS. Bold nucleic acid sequences represented the primers. The determined N-terminal amino acid sequences were marked in rectangular boxes.



	α helix	β strand
nAmb t 18	33.89%	16.78%
rAmb t 18	32.74%	22.18%

Figure 2. Purification and characterization of the recombinant and natural Amb t 18. (A) The rAmb t 18 was purified by Ni^{2+} affinity chromatography combined with anion exchange chromatography. Lane M, molecular weight marker; Lane 1, purified rAmb t 18. (B) SDS-PAGE analysis of purified nAmb t 18 and rAmb t 18. Lane M, molecular weight marker; Lane 1, purified nAmb t 18; Lane 2, purified rAmb t 18. (C) Circular dichroism analysis of nAmb t 18 and rAmb t 18.

Expression, purification and characterization of Amb t 18

The rAmb t 18 was produced in *E. coli* and expressed in supernatant contents. It was then purified by $Ni2^{+}$ affinity chromatography combined with anion exchange chromatography (**Figure 2A**). The nAmb t 18 was purified from the crude extracts of giant ragweed pollen by immunoaffinity chromatography using polyclonal antibodies against rAmb t 18 and was analyzed by SDS-PAGE (**Figure 2B**). Both nAmb t 18 and rAmb t 18 were confirmed by LC-MS/MS, and rAmb t 18 migrated to the position similar with its natural counterpart (**Figure 2B**). The sequence coverage of identified internal peptides of nAmb t 18 was 50.2% (**Figure 1**), and the rAmb t 18 was 37%. The first 15 N-terminal residue with determined as Gly-Arg-Lys-Phe-Phe-Val-Gly-Gly-Asn-Trp-Lys-Cys-Asn-Gly-Thr by Edman degradation. The nAmb t 18 and rAmb t 18 had similar circular dichroism spectra, in which the estimated contents of α-helix and β-strand in nAmb t 18 were 33.89% and 16.78%, while those from rAmb t 18 were 32.74% and 22.18%, respectively (**Figure 2C**).

Detection of the homologous Amb t 18 in the short ragweed pollen crude extracts

In Western blot, a specific positive band at about 26 kDa could be detected in the commercial pollen crude extracts of short ragweed by the anti-rAmb t 18 polyclonal antibodies (**Figure S2B**), this 26 kDa protein could be also visualized by SDS-PAGE and Coomassie blue G250 staining (**Figure S2A**).

IgE reactivity of sera from patients to Amb t 18

The ELISA results showed that 13 (35%) had a positive IgE response to the rAmb t 18 (**Figure 3A**), the serum sIgE level (the median and interquartile range (IQR)) of *Ambrosia* (kUA/L) from rAmb t 18-IgE positive sera (median = 6.31, IQR = 4.87-20.05 kUA/L) was higher than that of rAmb t 18-IgE negative sera (median = 4.43, IQR = 1.70-10.64 kUA/L) (**Table S1**). Both rAmb t 18 and nAmb t 18 could reacted with IgE in the four representative sera by Western blot (**Figure 3B**).

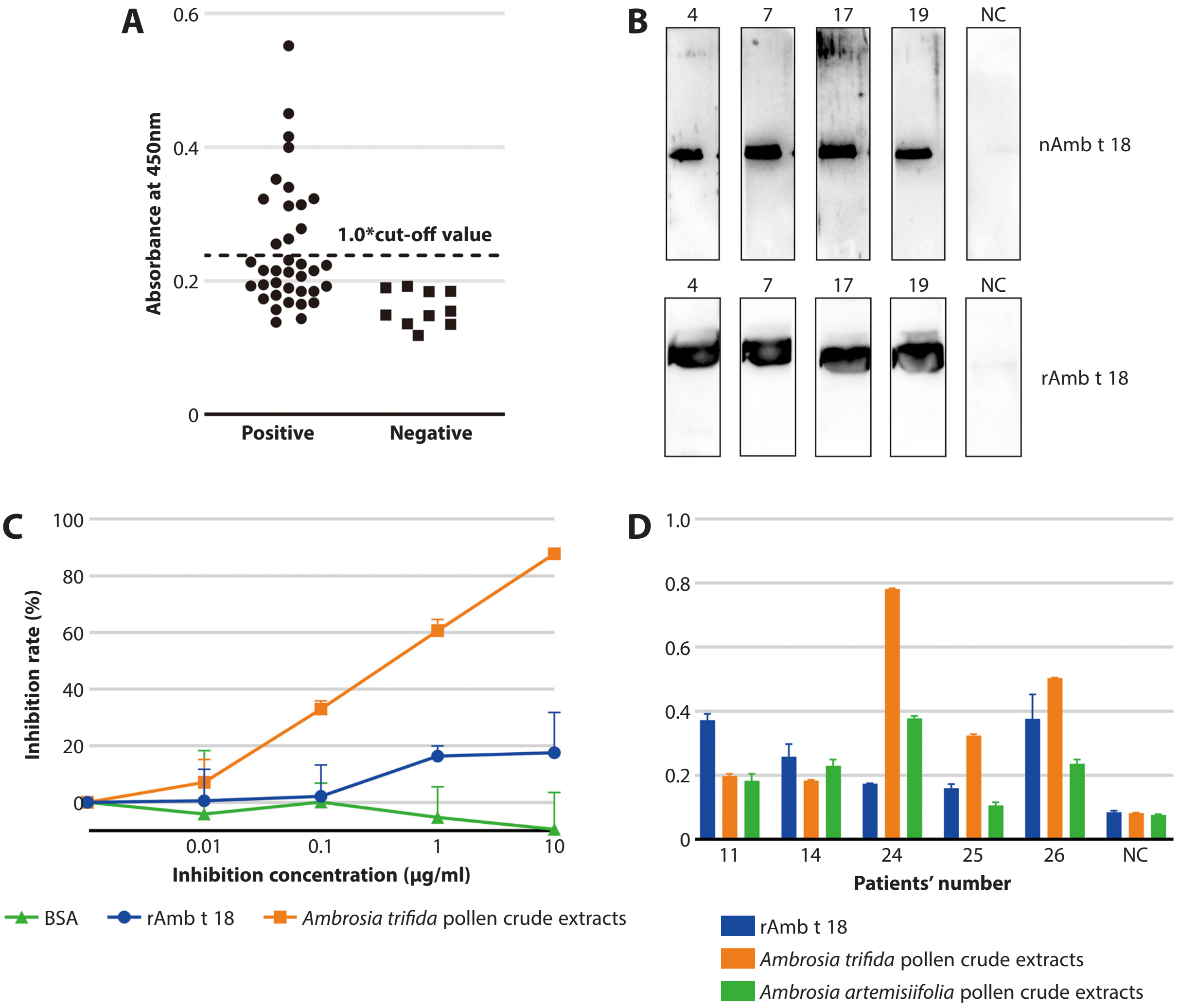


Figure 3. IgE reactivity of Amb t 18. (A) The IgE binding ability of rAmb t 18 was assessed by ELISA with 37 serum samples from *Ambrosia* pollen allergic patients and 10 non-allergic subjects. Each dot value is presented as the mean of duplicate wells. The cut-off value was calculated as average OD value + 3-fold SDs of the negative controls (cut-off: OD = 0.238). (B) IgE-Western blot analysis of nAmb t 18 and rAmb t 18. The number 4, 7, 17, 19 were the four samples with highest absorbance values in ELISA; NC, serum from nonallergic individual used as controls. (C) Inhibition of IgE binding to *Ambrosia trifida* pollen extracts by rAmb t 18. *Ambrosia trifida* pollen extracts and BSA was used as positive and negative controls, respectively. The data was presented as mean ± SD from triplicate wells. (D) Comparation of IgE reactivity of rAmb t 18, *Ambrosia trifida* and *Ambrosia artemisiifolia* crude extracts. The samples numbered 11, 14, 24, 25, and 26 in the figure tested positive in the ELISA; NC, 1 serum from health donor.

From the sera of 5 representative rAmb t 18-IgE positive patients, 2 had higher IgE-binding absorbance values against rAmb t 18 (0.3719 and 0.2581) than those against giant ragweed pollen crude extracts (0.1983 and 0.1840). Moreover, the IgE-binding absorbance values (mean ± standard deviation) in the same 5 sera against the giant ragweed crude extracts (0.3982 ± 0.2351) were higher than those against short ragweed (0.2265 ± 0.0945) (**Figure 3D**).

The IgE-binding to the crude pollen extracts was inhibited by 10 μg/mL of rAmb t 18 at an inhibition rate of 17.56%. (**Figure 3C**). In basophil activation test, the proportions (mean ± standard deviation) of CD63$^+$ basophils after rAmb t 18 stimulating were 14.5 ± 1.2, while those from unstimulated controls and anti-IgE stimulating were 8.9 ± 0.8 and 62.0 ± 6.4, respectively. By comparation, the rAmb t 18 at 10 μg/mL induced an approximate 1.4-fold to 1.9-fold increase in the proportion of activated basophils compared with PBS controls ($p < 0.001$) (**Figure 4 A-B**).

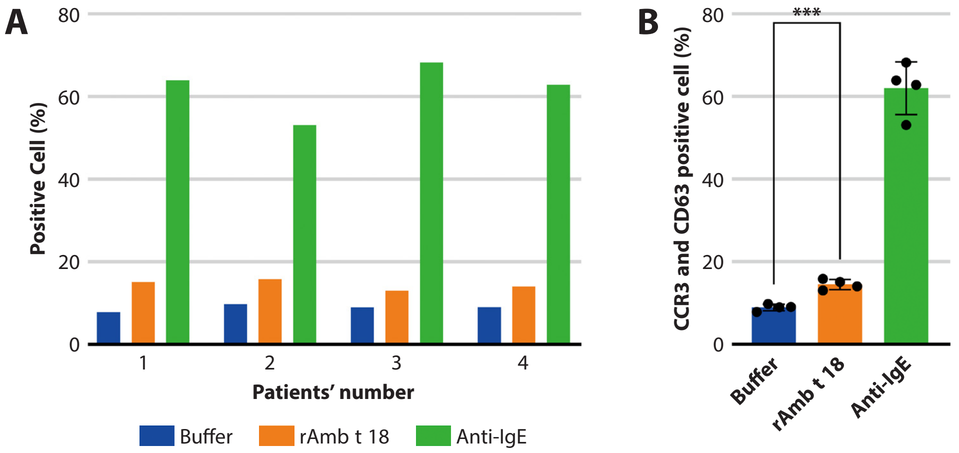


Figure 4. Basophil activation test. (A) The basophils were sensitized with four rAmb t 18 sIgE positive sera and stimulated with PBS, anti-IgE, rArt t 18 at final concentration of 10 µg/mL. (B) Comparation of the difference in stimulating rate between PBS and rAmb t 18 by paired-sample t-test (****p* < 0.001).

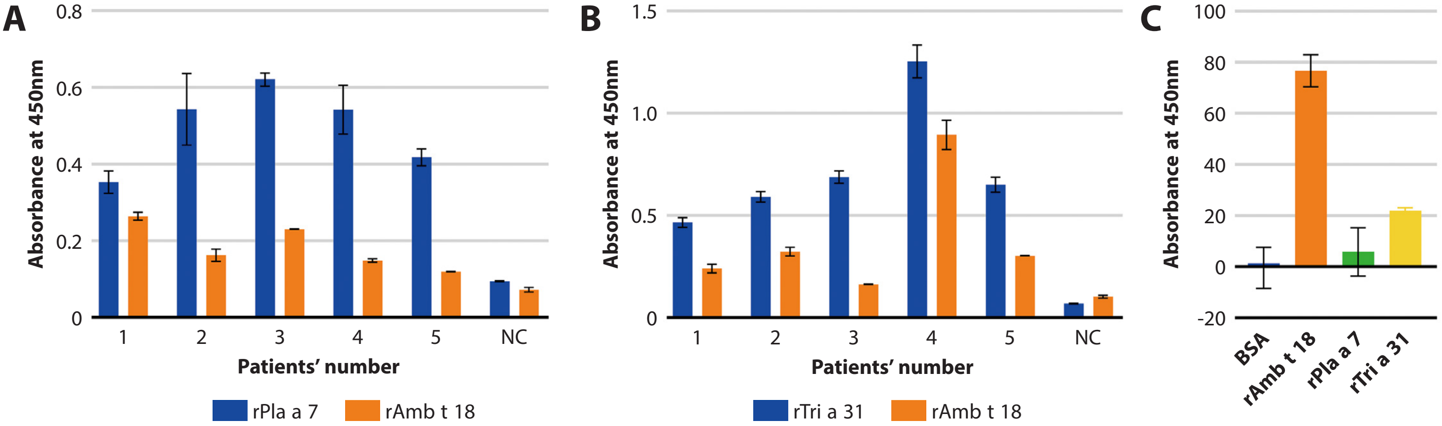


Figure 5. IgE cross-reactivity of Amb t 18. (A) Analysis of the IgE reactivity of rAmb t 18 by ELISA using 5 rPla a 7-positive patients' sera (No. 1-5) and 1 serum from healthy donor (NC). The rPla a 7 was used as a control. (B) Analysis of the IgE reactivity rAmb t 18 by ELISA using 5 rTri a 31-positive patients' sera (No. 1-5) and 1 serum from healthy donor (NC). The rTri a 31 was used as a control. (C) Inhibition of IgE binding to rAmb t 18 by rAmb t 18, rPla a 7 and rTri a 31 in a pool of rAmb t 18-IgE positive sera. BSA was used as a negative control.

IgE-cross reactivity of Amb t 18

Among rPla a 7-IgE positive sera, the IgE-binding absorbance values (mean ± standard deviation, SD) against the rPla a 7 (0.4953 ± 0.1092) were higher than those against the rAmb t 18 (0.1850 ± 0.0569) (**Figure 5A**), while the IgE-binding absorbance values against the rTri a 31 (0.7294 ± 0.2894) were also higher than those against the rAmb t 18 (0.3850 ± 0.2764) among rTri a 31-IgE positive sera (**Figure 5B**). The rAmb t 18, rPla a 7, and rTri a 31 achieved 76.70%, 5.80%, and 21.94% inhibition of IgE binding to rAmb t 18, respectively. (**Figure 5C**).

Conservation analysis and structure prediction of Amb t 18

Triosephosphate isomerase has been identified as allergens in food, mold and mites (**Figure 6A**). According to previous studies, triosephosphate isomerase had a total of four enzyme's active sites, and all of them were located in the conserved sequences.[31] We deduced that the possible four enzyme's active sites of Amb t 18 were ASN-10, LYS-12, HIS-96 and GLU-166 (**Figure 6B**), the RMSD value between Amb t 18 and Tri a 31 was lower than that of Pla a 7 (**Figure S3**). The heat map illustrated that triosephosphate isomerase is a highly conserved family of proteins, sharing a high degree of sequence identity even among species that are more distantly related in the phylogenetic tree (**Figure 6C**). The sequence alignment showed that the amino acid sequence of Amb t 18 exhibits 82% identity of *Platanus acerifolia* pollen allergen Pla a 7 and wheat allergen Tri a 31 (**Figure 6D**).

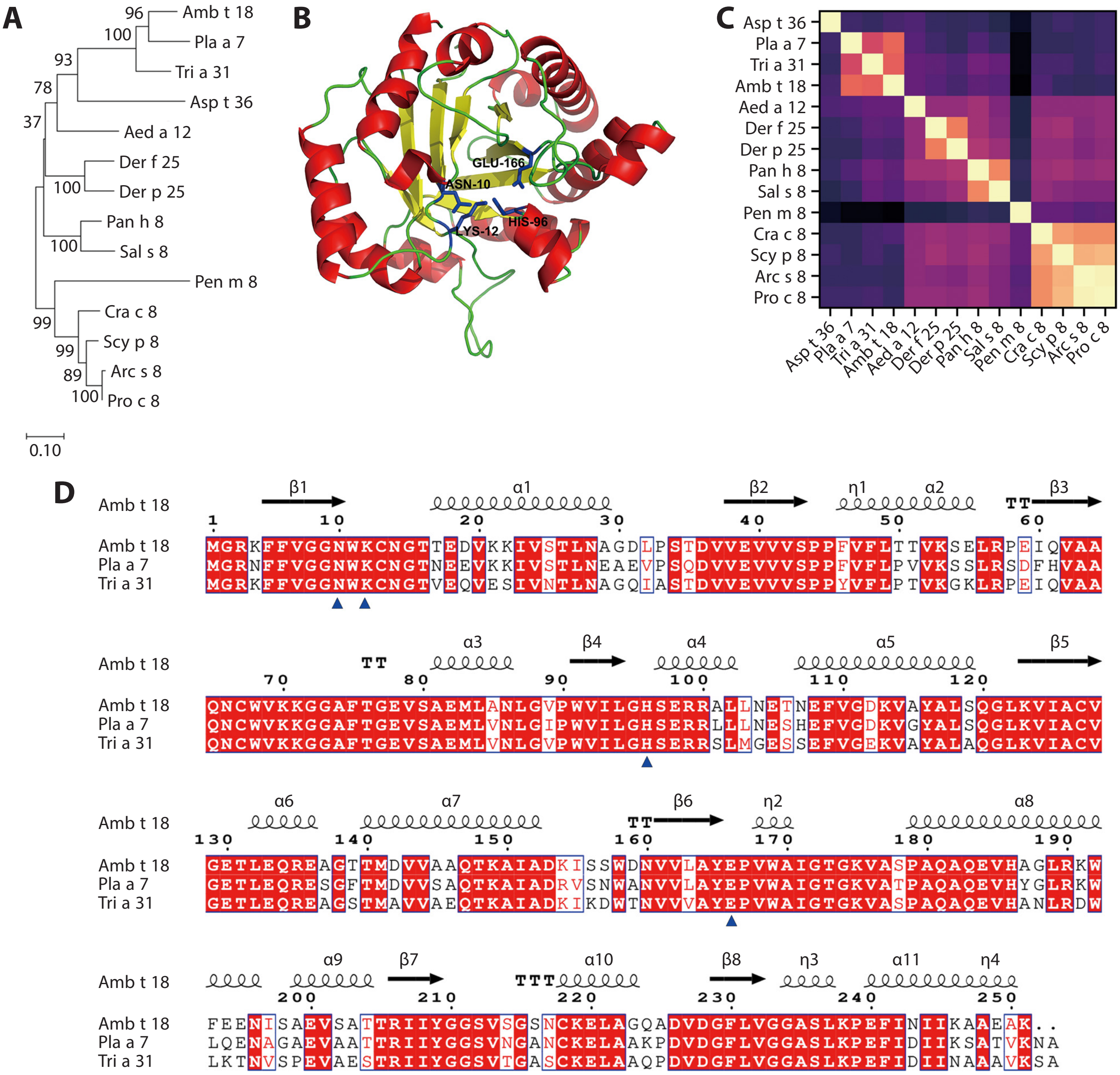


Figure 6. Amino acid sequence alignment of Amb t 18. (A) Phylogenetic analysis of Amb t 18 and other homologous allergens recorded in WHO/IUIS. (B) The 3D structure shown in cartoon mode. The four enzyme active sites are colored and labeled by black. Monomers were used as an illustration and surface characterization, in which the α-helix and β-strand are painted red and yellow, respectively. (C) The heat map of amino acid sequences of triosephosphate isomerase reported as allergens on the Official Allergen List: WHO/IUIS Allergen Nomenclature Subcommittee (http://www.allergen.org). (D) The sequence identity of Amb t 18 with homologous allergens from other plant species, in which Amb t 18 shared 82% with Pla a 7 and Tri a 31. The possible four active sites are highlighted by *filled triangle*.

Discussion

While allergic diseases cause a growing global health burden,[32] research on giant ragweed allergens remains limited. In this study, based on the preliminary results of the identified internal peptides from approximately 26 kDa IgE-binding band in *Ambrosia trifida* pollen extracts, the theoretical cDNA sequence was selected from the transcripts of next-generation sequence of *Ambrosia trifida* pollen mRNA. Primers were designed based on the theoretical cDNA for PCR cloning, despite the fact that the 5′-CAAATCTCATCACAAAGCTT-3′ primer was designed on the basis of the theoretical sequence, only the 5′-ACAAAGCTT-3′ segment proved functional, underscoring the necessity of PCR validation for next generation sequencing derived allergen transcripts.

Based on the cDNA sequence, the rAmb t 18 was expressed and purified from *E. coli* and nAmb t 18 was purified from pollen crude extracts by immunoaffinity chromatography. Both nAmb t 18 and rAmb t 18 were confirmed by LC-MS/MS. The sequence coverage of identified internal peptides of nAmb t 18 and rAmb t 18 were 50.2 % and 37%. The determined N-terminal residues were identical to that from the cloning result. Both the nAmb t 18 and the rAmb t 18 migrated to a similar position on SDS-PAGE, we noticed that the rAmb t 18 had a slightly higher molecular weight than the nAmb t 18, this may be due to the fused His-tag. The nAmb t 18 and rAmb t 18 had similar circular dichroism spectra, the little difference in spectra may be due to the existence of folding differences in partial regions of the recombinant protein. The CD spectra covering the 190–240 nm wavelength range are ideal for comprehensive secondary structure analysis,[33] we used the commonly applied 20 mM sodium phosphate buffer to recode the spectra, but the buffer exhibits strong absorbance below ~195 nm at concentrations 20 mM.[34] Since we observed high noise in the 190–200 nm range, we therefore restricted our analysis to 200–250 nm. Moreover, the previous report about K2D3 demonstrated the accuracy of data from 200–240 nm was equivalented to that from full 190–240 nm.[19] This property ensures the validity of α-helix and β-sheet content calculated from 200-240 nm.

Because it is hard to obtain enough natural allergen nAmb t 18, the rAmb t 18 was used in ELISA and inhibition ELISA tests. A total of 37 patients' sera with positive sIgE against ragweed pollen extract were selected based on the allergen code W1 in the ImmunoCAP system, since it is the only China Food and Drug Administration (CFDA)-approved material for ragweed-sIgE test. The previous study also suggested that short ragweed could be used as the representative species for diagnosis among the related ragweed species.[35] In the ELISA test, 13 out of 37 (35.1%) patients' sera showed a positive IgE response to rAmb t 18. A similar IgE-binding pattern could be observed between nAmb t and rAmb t 18 in Western blot. Due to the limit loading amount of nAmb t 18, the bands for the nAmb t 18 appeared thinner IgE binding signal. Nevertheless, the IgE-binding profiles were consistent between the two molecules. In pooled rAmb t 18 IgE-positive sera, 17.56% of the IgE-binding to the pollen extracts could be inhibited by rAmb t 18 at 10 μg/mL. Because the lack of fresh whole blood cells necessary for the direct BAT, we were only able to use the indirect BAT to investigate the cell-activating function of Amb t 18. We also acknowledge that a direct comparison of the IgE-reactivity and basophil activation among nAmb t 18, rAmb t 18 and the whole pollen extract is essential for a complete evaluation, we will be committed to solve the limited availability of nAmb t 18 to evaluate their value in component-resolved diagnostics (CRD) in subsequent studies. The allergenicity results suggested the triosephosphate isomerase in giant ragweed may be a minor allergen. Compared with our reported the same protein family allergen (Pla a 7) from *Platanus acerifolia* pollen, the IgE-binding rate of Amb t 18 (35.1%) is closer to that of Pla a 7 (41.4%),[16] which meant triosephosphate isomerase allergens may play similar roles in tree and weed pollen. Nevertheless, Amb t 18 had the ability in increasing the crude extracts-based diagnostic sensitivity in some patients based on the comparation of their IgE-reactivity. Moreover, the IgE-reactivity against giant ragweed pollen extracts was higher than that against short ragweed pollen extracts among Amb t 18 IgE-positive patients, which may be helpful for distinguishing the sensitization of giant ragweed and short ragweed. Of course, this requires further verification of its clinical performance through component-resolved diagnosis. Our study provides a clue and a useful molecule for allergens panel in this regard.

Triosephosphate isomerase is an enzyme that interconverts the 3-carbon sugars.[36] Currently, 13 allergens belonging to triosephosphate isomerase have been recorded in the IUIS database. They are highly conserved and can be classified by source (fungi, plants, animals) and by route of sensitization (airway, injection, food), for example Tri a 31 and Pla a 7 in plants,[13,16] Der p 25 and Der f 25 in mites,[15] Asp t 36 in fungus,[14] Pan h 8 and Sal s 8 in fishes,[37] and Cra c 8, Scy p 8, Arc s 8 and Pro c 8 in shrimp and crab.[38,39] Based on the polyclonal antibodies against rAmb t 18, its homolog could be also detected in short ragweed. Based on the alignment of their amino acid sequence, Amb t 18 exhibited the highest sequence identity (82%) with Tri a 31 and Pla a 7 compared with 44%-62% sequence identity with the other homologous allergens. This phenomenon also occurs in other pollen allergen protein families. For example, our group found new type of pollen allergens belonging to the fructose-bisphosphate aldolase family (Art an 14 and Art si 14), the sequence identity between them is as high as 99%,[20] and the sequence identity between Art si 14 with the fish food allergens Sal s 3 and Pan h 3 reaches 60%-62%. Similar characteristic could be also observed from enolase allergens.[40] This property may make them a specific type of conserved allergen family, the clinical implications of patients sensitized to such allergens would be an interesting target for future exploration, especially the cross-reaction.

The results about IgE-cross reactivity indicated Amb t 18 could react with the sera from patients allergic to *Platanus acerifolia* or *Triticum aestivum*, but it is limited. The usual view has been that cross-reactivity requires more than 70% sequence identity.[41] Moreover, the similarity in the 3-dimensional folding pattern is also a critical factor for cross-reactivity.[42] Although Amb t 18 shared high sequence identity with Tri a 31 and Pla a 7, the sequence variations may alter the conformational epitopes for IgE binding. For example, IgE antibodies from goat's milk (GM)-allergic/cow's milk (CM)-tolerant patients recognize caprine β-casein without cross-reacting with bovine β-casein despite 91% sequence identity.[43] This indicated that high linear homology doesn't always lead to functional IgE cross-reactivity. The RMSD results showed that Amb t 18 is structurally more similar to Tri a 31, which may also explain why the inhibition rate for Tri a 31 is higher than for Pla a 7. Moreover, the rAmb t 18-sIgE positive patients have a clear clinical history of allergic symptoms in late summer and autumn, whereas Pla a 7 is an allergen from spring pollen (plane tree) and Tri a 31 is from wheat flour, such low clinical relevance may also be the reason for the low IgE-cross reactivity of these molecules in the patients of the study. The limited cross-reaction also indicated the IgE-binding of Amb t 18 to patients' sera IgE was specific reaction.

Conclusion

In summary, we identified and characterized Amb t 18 as a novel allergen in *Ambrosia trifida* pollen. It is the first triosephosphate isomerase allergen found in weed pollen. This finding is crucial for enriching information on the allergenic composition of giant ragweed pollen, as well as expanding knowledge of the triosephosphate isomerase allergen and paving the way for diagnostic and therapeutic strategies for ragweed pollen allergy.

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Authors' Contributors

- JFW, JLS, and ZQX designed and guided this study.
- YFX, KL, LXZ, YLC and YSY contributed to data collection, YFX wrote the initial draft with all authors providing critical feedback and edits to subsequent revisions.
- JFW and ZQX revised the manuscript.

Conflict of interest

The authors declare that they have no known competing interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary material

Ragweed pollen and patients' sera

The commercial pollen crude extracts of short ragweed were dissolved in phosphate buffered saline (PBS). All patients had sIgE to ragweed pollen (> 0.35 kU/L) based on ImmunoCAP system (ImmunoCAP code: W1) as we previously reported (**Table S1**).[1] The recombinant Pla a 7 (rPla a 7) and recombinant Tri a 31 (rTri a 31) were prepared in-house as our previously reported method.[2] The 5 rPla a 7-IgE positive sera and 5 rTri a 31-IgE positive sera were screened from patients allergic to *Platanus acerifolia* or *Triticum aestivum* by ELISA and used for testing the cross-reactivity with Amb t 18. Most (35 of 37) of the ragweed pollen allergic patients had a clear history of allergic symptoms in late summer and autumn, and 2 patients suffered rhinitis with indefinite seasons. Patients had not only solely asthma or allergic rhinitis or both were described as other symptoms. Moreover, only 2 patients were willing to undergo skin prick test against the extracts of *Ambrosia trifida* pollen, with induced wheal ranged from 5*5 (+) to 5*6 (+) and flare ranged from 25*20 (+) to 10*15 (+), others were not willing to do so. Therefore, we have to rely on the ImmunoCAP results combined with their symptoms history as the selection criteria for ragweed pollen allergic patients.

The crude extracts of giant ragweed pollen were prepared by mixing the pollen with 10 volume (w/v) of 10 mM phosphate in 1 × PBS and a final concentration of 1 mM phenylmethylsulfonyl fluoride (Sangon, Shanghai, China) at 4°C overnight as described previously.[1] The IgE-binding bands in giant ragweed crude extracts were screened by IgE-Western blot based on the pooled sera of *Ambrosia* pollen allergic patients as our reported previously.[3]

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Table S1. Clinical characteristics of Amb t 18-positive and negative allergic patients in the study.

Characteristic	Positive Total = 13	Negative Total = 24
Age, mean ± SD (Range)	25.4 ± 11.18 (6-38)	31.4 ± 14.63 (9-62)
Age, n (%)		
Children (< 18 years)	5 (38.46%)	5 (20.83%)
Adults (≥ 18 years)	8 (61.57%)	19 (79.17%)
Gender, n (%)		
Male	8 (61.57%)	10 (41.67%)
Female	5 (38.46%)	14 (58.33%)
Diagnosis, n		
Allergic Rhinitis	5	6
Allergic Rhinitis and Asthma	3	7
Asthma	0	1
Allergic conjunctivitis	0	1
Allergic Rhinitis and allergic conjunctivitis	2	2
Other	3	7
Serum sIgE level of *Ambrosia artemisiifolia* (kUA/L), median (IQR)	6.31 (4.87-20.05)	4.43 (1.70-10.64)

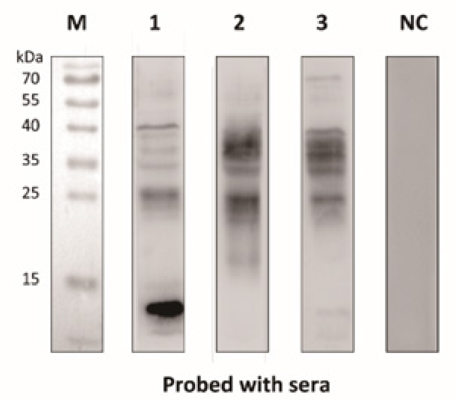

Figure S1. Analysis of the IgE-binding bands in giant ragweed pollen crude extracts by IgE-Western blot. M, standard maker; No. 1-3, three different sera pools from ragweed pollen allergic patients; NC, serum from healthy donor.

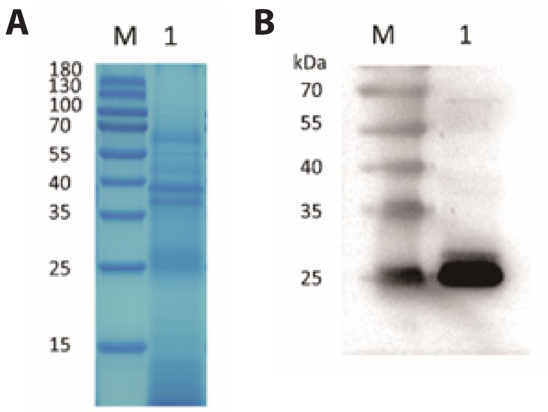

Figure S2. Detection of the expression of Amb t 18 in short ragweed pollen. (A) *Ambrosia artemisiifolia* pollen crude extracts were visualized by SDS-PAGE and Coomassie brilliant blue G250 staining. Lane M, standard marker; Lane 1, *Ambrosia artemisiifolia* pollen crude extracts. (B) Detection of the homologous Amb t 18 in the short ragweed pollen crude extracts by Western blot and anti-rAmb t 18 polyclonal antibodies. Lane M, standard marker; Lane 1, the homologous allergen of Amb t 18 in short ragweed.

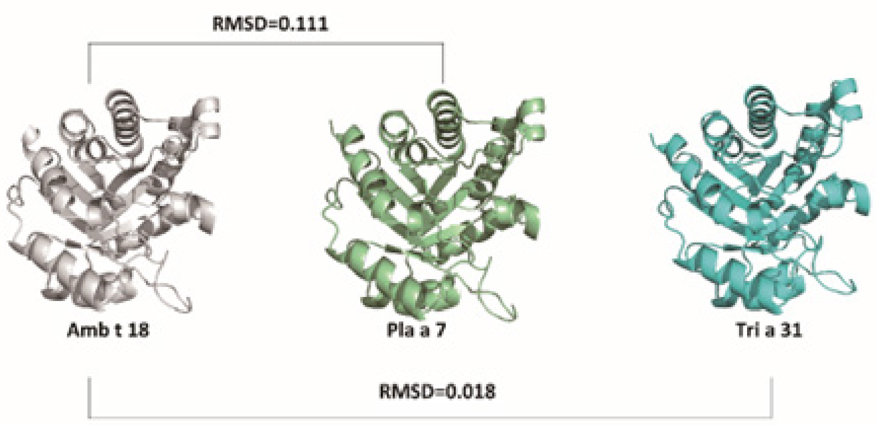

Figure S3. Three protein three-dimensional structural models (Amb t 18, Pla a 7, Tri a 31) and the corresponding RMSD values between each other.