



Production of a chimeric allergen derived from the major allergen group 1 of house dust mite species in *Nicotiana benthamiana*

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ABSTRACT

Plants are widely accepted as a general platform for the large-scale production of recombinant proteins, which has been demonstrated by the successful expression of various exogenous proteins. Using plants as a bioreactor for mass production of target proteins for vaccines is thought to show the most potential. This study explores whether a chimeric allergen R8, derived from the major allergen group 1 of house dust mites species (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*), is expressed in tobacco. The highly efficient and useful *Tobacco mosaic virus* RNA-based overexpression (TRBO) vector was used to investigate expression of the R8 molecule in tobacco by agroinfection. Presence of R8 was detected using SDS–PAGE and Western blotting. Purified allergens were characterized using IgE-binding activity assay and allergen-specific immunotherapy (ASIT) in murine asthmatic models. The recombinant R8 was successfully expressed in tobacco leaves. The pro-peptide was observed in the herbaceous leaf extracts. This protein exhibits properties similar to the parental allergen ProDer f 1 expressed in *Escherichia coli* or tobacco with respect to IgE immunoreactivity. R8 also rectifies imbalance of TH1/TH2 cells. An herbaceous plant expression system model allows mass production of R8, which might be used in the future for diagnosis of asthma or production of a candidate vaccine for allergen-specific immunotherapy of asthma.

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1. Introduction

Type I IgE-mediated allergy, characterized by hypersensitivity to allergen(s), is becoming an important global health problem [1], and more than 25% of the population in industrialized countries suffered from allergic diseases such as asthma [2,3]. Even worse, incidence of allergic diseases seems to be rising [1,4,5]. Symptomatic anti-inflammatory treatment using different drugs such as antihistamines and/or corticosteroids may alleviate the clinical symptoms, but is not curative. Thus, it is urgent to expect certain new treatment strategies for allergic disorders.

Allergen-specific immunotherapy (ASIT) is a well-established way to modulate the immune process during the pathogenesis of allergic disease, and has clinically safe, effective and long-lasting outcomes [3,6,7]. Conventional ASIT utilizes crude allergen extracts developed from allergen source as vaccines, administered by repeated subcutaneous injections of increasing doses; this treatment is sometimes accompanied by severe side-effects, such as anaphylaxis [8–10].

Bioreactors by herbaceous plant models are considered an alternative to highly purified allergens as vaccines for ASIT and have been developed recently [11–19]. Compared with natural allergens, the production of recombinant proteins in plants offers several advantages: (1) ability for consistent mass production, (2) a better definition and easier standardization compared to crude allergen extracts, (3) a native conformation to mimic natural allergens [20–25], and (4) absence of bacterial toxins or animal pathogens, making them suitable for pharmaceutical use. Successful production of several biopharmaceutical proteins for human health in plants has been reported [1,16,19,26–30].

As one of transient expression systems, *Tobacco mosaic virus* (TMV), a rod-shaped virus with a single-stranded plus-sense RNA genome, is modified to express foreign interested genes wide spreadly by inoculating with plants through agroinfection. However, there are several notable disadvantages of using general plant virus-based transient expressed systems, such as post-transcriptional gene silencing of target genes [31], high concentration of *Agrobacterium tumefaciens* infiltrating into leaves to get highest expression levels possible [32], and low agroinfection efficiency of typical 35S promoter-driven TMV vectors [33]. An improved agroinfection-compatible TMV vector is available, named TMV RNA-based overexpression (TRBO) vector, which lacks the coding sequencing of the coat protein gene in TMV

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virus [34]. TRBO overcomes the shortcomings described above and is mainly characterized by highly efficient agroinfection, high foreign protein expression levels and inability to form virus particles in hosts. Based on these advantages, TRBO was selected as vector in our study.

Previously, we applied DNA shuffling to two group 1 mite allergen genes, i.e., one group 1 allergen from dust mite *Dermatophagoides farinae* (ProDer f 1) and another from dust mite *Dermatophagoides pteronyssinus* (ProDer p 1). A chimeric gene R8 was screened successfully and encoded a protein characterized with hypoallergenicity and high T-cell immunogenicity [35]. The present study aimed to investigate the expression of an *Agrobacterium*-mediated gene transfer expression vector that would increase the stable expression of R8 in tobacco for research or production purposes. Additionally, we also used murine asthmatic models to compare the predicted characterization of R8 with its parent ProDer f 1 protein for tentative diagnosis or ASIT of asthma.

2. Materials and methods

2.1. Animals

Female BALB/c mice (6 weeks of age) were purchased from the Center for Comparative Medicine, Yangzhou University (License No.: SCXK 2007-0001) and provided with food and water *ad libitum* under specific-pathogens free conditions. All procedures were approved by the Animal Research Ethics Board of Wannan Medical College.

2.2. Tobacco plant

Nicotiana benthamiana plants were grown in a growth illumination chamber (MLR-351H, SANYO Electric CO. Ltd, Japan) with a 16-h photoperiod, 25–27 °C.

2.3. Genes encoding allergens

The chimeric allergen gene R8, derived from two major allergen genes, ProDer f 1 (GenBank No. AB034946.1) and ProDer p 1 (GenBank No. U11695.1) served templates as DNA shuffling according to the protocols described by Stemmer [36], and its amino acid sequence was characterized with reserved T cell epitopes and decreased B cell epitopes [35].

2.4. Recombinant binary plasmid construction

The full-length ProDer f 1 coding sequence (GenBank No. AB034946.1) and chimeric R8 was amplified by PCR (sense strand primer: 5'-CGT CCA GCT TCA ATC AAA ACT-3'; antisense strand primer: 5'-TCA CAT GAT TAC AAC ATA TGG-3'). *Pac* I and *Not* I restriction sites were inserted at the 5' and 3' ends, respectively. PCR amplifications were carried out in a total reaction volume of 50 µl, containing 10 ng cDNA, 1 × PCR buffer (50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl pH 9.0), 0.2 mM dNTP, 0.4 µM primer (the sense and antisense strand primers described previously), and 2.5 units *Taq* DNA polymerase. For PCR analysis, denaturation was performed at 94 °C for 4 min, followed by 35 cycles, carried out as follows: 94 °C for 30 s; 58 °C for 30 s; and 72 °C for 1 min; with a final elongation at 72 °C for 10 min. The 912 bp PCR product was digested with *Pac* I-*Not* I and cloned into the *Pac* I-*Not* I-digested TRBO to create recombinant plasmids TRBO-ProDer f 1 and TRBO-R8 (Fig. 1). Nucleotide sequence was confirmed by DNA sequencing.

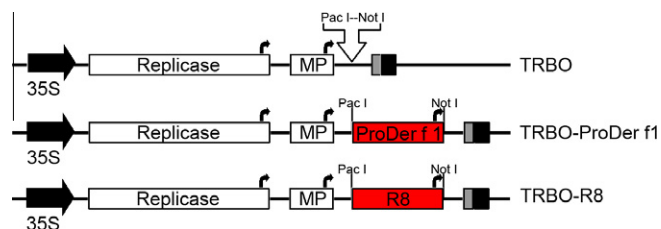


Fig. 1. Structures of TRBO-ProDer f 1 and -R8 expression vectors used in this project. The T-DNA regions of binary plasmids used in this project are represented. Black arrow: CaMV duplicated 35S promoter; black box: CaMV polyA signal sequence/terminator; dark gray box: ribozyme; bent arrows: subgenomic promoters. ORFs are represented by white or red boxes. Replicase, TMV 126 K/183 K ORF; MP, movement protein. (For interpretation of color in this figure, the reader is referred to the web version of this article).

2.5. Agroinfection

The recombinant binary vectors TRBO-ProDer f 1 and TRBO-R8 purified from *E. coli* culture were transferred into *Agrobacterium tumefaciens* (strain GV3101) by the freeze-thaw method [37]. Transformed *A. tumefaciens* were placed on Luria-Bertani (LB) plates with 50 µg/ml Kanamycin, 25 µg/ml gentamycin and 10 µg/ml rifampicin for plasmid screening. Individual colonies of *A. tumefaciens* transformed with a binary plasmid were grown to an OD₆₀₀ of 1.0 in liquid LB media supplemented with 10 mM MES, pH 5.7, 50 µg/ml Kanamycin, 25 µg/ml gentamycin and 20 µM acetosyringone. Cells were collected by centrifugation and resuspended in 10 mM MES, pH 5.7, 10 mM MgCl₂, and 200 µM acetosyringone [38]. For transient expression, tobacco plants (*N. benthamiana*) were agroinfiltrated according to Wydro et al. [39]. Cells sat at room temperature in induction media for 2–24 h before infiltration into the abaxial surface of *N. benthamiana* leaves using a 1 ml syringe with no needle. Plants were harvested and weighed daily from the 3rd to 7th day of incubation. Recombinant proteins were extracted as described below.

2.6. Total protein extraction

ProDer f 1 and R8 were expressed in plants from a TRBO replicon by agroinfection. The leaves collected from both types of transgenic tobacco plant tissues (described above) were ground in liquid nitrogen. Samples were extracted using four volumes (per gram fresh weight) of extraction buffer (50 mM phosphate, pH 8.0, 10 mM Tris, pH 8.0, 500 mM NaCl, 0.1% Tween-20, 0.1% Nonidet P-40, 0.1% β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride). Total soluble proteins were centrifuged at 12,000g, 4 °C for 20 min, resulting in clarified supernatant.

2.7. Purification of His6-tagged ProDer f 1 protein

The procedure was performed according to the description given by Lindbo [34]. Briefly, the clarified supernatant containing His6 C-terminally tagged ProDer f 1 or R8 protein underwent a –20 °C freeze-thaw cycle. After thawing, samples were centrifuged (as before). Ten mM imidazole (final concentration) was added to the supernatant. One-half milliliter of washed Ni-NTA His-Bind® Resins (Invitrogen, Carlsbad, CA, USA) was added to 8–10 ml of extract and incubated at 4 °C on a rocker for about 1.5 h. The column was washed with 20 column volumes of wash buffer (50 mM phosphate, pH 8.0, 500 mM NaCl, 0.1% Tween-20, and 20 mM imidazole). Bound His-tagged ProDer f 1 protein was eluted using 250 mM imidazole in 1 × phosphate-buffered saline (PBS, 11.9 mM phosphate, pH 7.4, 137 mM NaCl, 2.7 mM KCl, pH 7.2). The eluted fraction was dialyzed twice (6 h to overnight) into

1000 volumes of $1 \times$ PBS at 4°C . Protein concentration was estimated using Quick Start™ Bradford protein assay (Bio-Rad, Berkeley, CA, USA), with bovine serum albumin as the standard.

2.8. SDS-PAGE

Equal volumes (about 20 μg of total soluble proteins) of clarified extract of each treatment were analyzed on a 12.5% polyacrylamide gel according to Laemmli's method [40] in a Mini-PROTEAN 3 system (Bio-Rad) and stained with Coomassie blue R-250 (Sigma-Aldrich® Co. LLC., St Louis, MO, USA) to visualize the proteins.

2.9. Western blot analysis

For western blot analysis, 20 μg of total soluble proteins obtained from plant extracts were separated by 12.5% SDS-PAGE and transferred onto an Immobilon-P membrane (EMD Millipore, Billerica, MA, USA). Membranes were incubated in blocking buffer (5% dried milk, 0.5% Tween-20 in PBS, pH 7.2) for at least 30 min. Afterward, the membranes were incubated for 2 h in blocking buffer containing ProDer f 1-specific rabbit polyclonal antiserum (obtained after immunization with the purified native Der f 1 protein) mixed with PBS at a ratio of 1:10,000. A horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma-Aldrich® Co. LLC.) mixed with PBS at a ratio of 1:10,000 was used as secondary antibody followed by three washes in blocking buffer (20 min each). Transferred proteins were visualized using DAB Horseradish Peroxidase Color Development Kit (Sangon Biotech, Shanghai, China) in PBS (pH 7.2) according to the manufacturer's instructions. The yield of R8 expressed in tobacco tissue was also quantified using purified recombinant His6-tagged R8 to generate a standard curve.

2.10. IgE-binding activity assay

IgE-binding activity assay was measured by Enzyme Linked Immunosorbent Assay (ELISA). Twenty-five serum samples were obtained from *D. farinea*-allergic patients in the Yijishan Hospital of Wannan Medical College. The Medical Ethics Committee of Wannan Medical College approved collection of the samples. Immunoplates were coated overnight with rProDer f 1 (expressed in *Escherichia coli*), tProDer f 1 (expressed in tobacco) or tR8 (R8, expressed in tobacco) (500 ng/well) at 4°C . Plates were then washed 5 times with 100 μl per well of Tris-buffered saline (TBS)-Tween buffer (TBST, 50 mmol/L Tris, pH 7.5; 150 mmol/L NaCl; 0.1% Tween-20) and blocked for 1 h at 37°C with 150 μl of TBST supplemented with 1% BSA (Sigma-Aldrich® Co. LLC.). Sera from 25 patients allergic to *D. farinea* diluted in TBST buffer at a ratio of 1:8 (all of the individual sera had a RAST value >100 kU/L) were then incubated for 1 h at 37°C . Plates were washed 5 times with TBST buffer; the allergen-IgE complexes were detected after incubation with HRP-sheep anti human IgE (Sigma-Aldrich® Co. LLC.) (dilution 1:1000 in TBST buffer) at 37°C for 2 h. The plate was then washed 5 times with TBST, and 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution was added, allowing it to react 20 min at 37°C . Stop buffer of 50 μl for each well was immediately used to terminate the reaction. $A_{450\text{ nm}}$ was measured in an ELISA Reader (BioTek, Winooski, VT, USA).

2.11. Development of mouse models with asthma and ASIT

Seventy-five BALB/c mice were randomly assigned to five groups ($n = 10$ for each), i.e., PBS group, asthma group, rProDer f 1 group, tProDer f 1 group and tR8 group. On days 0, 7 and 14, mice were intraperitoneally injected with 10 μg relevant allergen, respectively, which was dissolved in 100 μl PBS containing 2%

(W/V) $\text{Al}(\text{OH})_3$ suspension. The PBS group received PBS injection instead. At day 21, the animals were caged in the airway challenge apparatus, and challenged by nebulized inhalation of allergen suspension (0.5 $\mu\text{g}/\text{ml}$) for 30 min on 7 successive days. The PBS group was challenged by PBS instead. The mice in rProDer f 1, tProDer p 1 and tR8 groups underwent ASIT by intraperitoneal injection of rProDer f 1, tProDer f 1, and tR8 (100 $\mu\text{g}/\text{ml}$), respectively in doses of 200 μl . ASIT was conducted daily for 30 min prior to the inhalation treatment on days 25–27. The PBS group experienced both intraperitoneal injection and aerosol inhalation of PBS. The asthmatic group received no treatment.

2.12. Histopathologic examination of the pulmonary tissues

The pulmonary sections were obtained from the other side of the lung free of lavage, fixed in 10% formalin overnight, embedded in paraffin, sliced conventionally and stained with hematoxylin and eosin (HE). The inflammatory changes were examined microscopically and assessed based on the extent of eosinophils infiltration, epithelia damage, and edema in the lung, according to the scoring protocol described by Underwood [41].

2.13. Detection of cytokines in BALF and antibodies in sera

Twenty-four hours after the final aerosol challenge, the mice were anesthetized with intraperitoneal injection of 100 μl 0.5% pentobarbital sodium. After the trachea of each mouse was annulated, a syringe with 19-gauge needle was used to infuse 0.3 ml of sterilized PBS and withdraw bronchoalveolar lavage fluid (BALF). This was repeated two more times, and a total of 0.9 ml BALF was obtained per mouse. Subsequently, BALF was centrifuged at 3000g for 5 min at 4°C , and the supernatant was collected and stored at -80°C . The blood samples were also collected via orbital cavity, centrifuged by 4000g at 4°C for 5 min and stored at -80°C . ELISA was performed to detect the levels of IFN- γ , IL-2, IL-4 and IL-10 in BALF, as well as serum antibodies of IgE, IgG₁ and IgG_{2a}, according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA).

2.14. Statistical analysis

Statistical analysis was carried out using SPSS for Windows, version 16.0 (SPSS, Chicago, IL, USA), and the statistical data for each group were expressed in ($\bar{x} \pm s$) in terms of one-factor analysis of variance. The group comparisons were performed using least significant difference-*t* (LSD-*t*) and Tamhane's T_2 method. A *P*-value of less than 0.05 was accepted as significant.

3. Results

3.1. Transient expression and purification of R8

The TRBO vector was modified by inserting the sequence encoding for R8 and the resulting plasmid TRBO-R8 was used to infect *N. benthamiana* plants. Three days later, leaves from the herbaceous plants showing local infection were collected and subjected to further analysis. Total soluble protein extracted from transgenic plant leaves expressing R8-specific transcripts and control lines was subjected to SDS-PAGE and western blot analysis. In the transgenic plant, an R8-specific signal of the correct length was detected (lane 2–6 in Figs. 2 and 3A). There was no detectable signal in the healthy controls (lane 1 in Figs. 2 and 3A).

tR8 was expressed as a C-terminally His6-tagged protein and was purified to homogeneity by nickel affinity chromatography under denaturing conditions. After refolding by successive dialysis

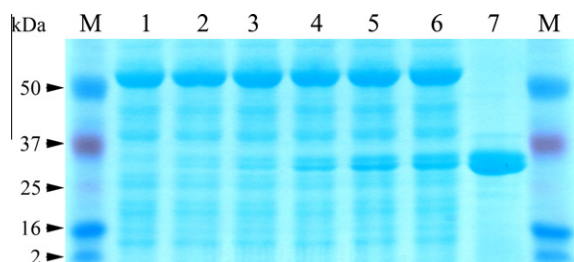


Fig. 2. SDS-PAGE analysis of chimeric protein expressed in tobacco leaves. In each lane, 20 μ g of extracted protein were loaded. M: protein Marker; lane 1: negative control (TRBO vector infected plant leaves); lanes 2–6: total protein in tobacco leaves harvested at 3, 4, 5, 6, 7d after infiltration; lane 7: purified R8 from tobacco leaves.

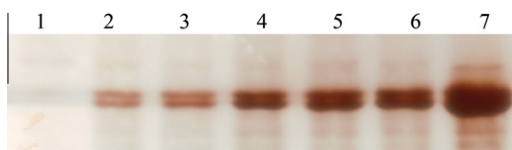


Fig. 3. Western blot analysis and quantification of R8. A. Western blot analysis of total proteins from TRBO-R8 infected plant leaves. In each lane, 20 μ g of extracted protein were loaded. Lane 1: negative control (TRBO vector infected plant leaves); lanes 2–6: total protein in tobacco leaves harvested at 3, 4, 5, 6, 7d after infiltration; lane 7: purified R8 from tobacco leaves. B. Quantification of R8. The yield of R8 was assessed as described in Section 2.

against decreasing concentrations of urea, purified R8 migrated as a single band at 35 kD on SDS–PAGE (lane 7 in Fig. 2). The band was recognized with rabbit polyclonal antibodies raised to Der f 1, which confirmed that R8 was homologous with parental allergen rProDer f 1 (lane 7 in Fig. 3A). After quantification, the yield of R8 changed from 0 mg/g at 0 d to 4.54 ± 0.142 mg/g at 7 days (Fig. 3B).

3.2. tR8 from tobacco retains IgE-binding reactivity toward allergic sera

The sera were tested by ELISA using plates coated with equivalent amounts of rProDer f 1, tProDer f 1 or R8. Results showed the extent to which the tR8 produced in *N. benthamiana* retained the capacity to bind IgE from the sera of *D. farinea*-allergic patients. The results demonstrate that the IgE-binding reactivity of R8 (1.45 ± 0.47) was similar to that of rProDer f 1 (1.44 ± 0.47 , $P = 0.624$) or tProDer f 1 (1.39 ± 0.35 , $P = 0.888$) (Fig. 4).

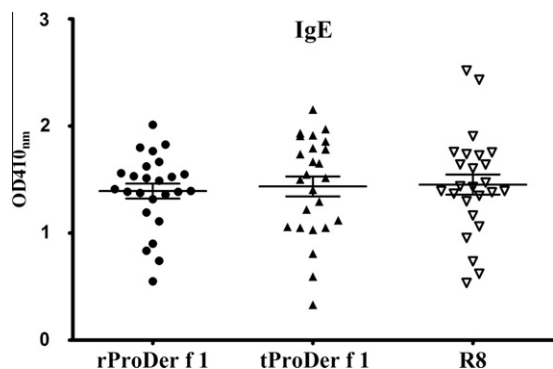


Fig. 4. IgE reactivity of R8 molecules expressed in tobacco plant. IgE reactivity was assessed as described in the Materials and methods section, using sera from 25 house dust mite (HDM)-sensitized patients. IgE in sera binding with R8 expressed in *N. benthamiana*, rProDer f 1 expressed in *E. coli* and tProDer f 1 expressed in *N. benthamiana*.

3.3. Evaluation of the prophylactic potential of R8 in sensitized mouse model

To verify the effects of R8 for ASIT on the asthmatic model animals, ELISA was performed to detect the levels of IFN- γ , IL-2, IL-4 and IL-10 in BALF as well as serum antibodies of IgE, IgG₁ and IgG_{2a}. Compared with the PBS group (141.77 ± 42.58 pg/ml), mice immunized by R8 produced a higher level of IFN- γ (459.54 ± 49.45 pg/ml) than those of the asthma group (53.31 ± 20.93 pg/ml), rProDer f 1 group (302.13 ± 45.13 pg/ml) and tProDer f 1 (295.33 ± 35.56 pg/ml) group ($P < 0.01$, Fig. 5A). Similar trends were seen regarding the levels of IL-2 and IL-10 ($P < 0.01$, Fig. 5B and D). In contrast, animals vaccinated with R8 allergen produced a lower level of IL-4 (40.48 ± 6.68 pg/ml) than the asthma group (88.91 ± 13.25 pg/ml) ($P < 0.01$, Fig. 5C). However, the IL-4 level was still higher than that of PBS group (11.09 ± 4.65 pg/ml) ($P < 0.01$, Fig. 5C). There was no statistical difference between rProDer f 1 and tProDer f 1 groups ($P > 0.05$) at the levels of cytokines, including IFN- γ , IL-2, IL-4 and IL-10.

The resulting serum IgE, IgG₁ and IgG_{2a} antibody levels suggested that the IgE level in tR8 group (48.80 ± 5.94 μ g/ml, $P < 0.01$), rProDer f 1 group (63.20 ± 5.64 μ g/ml, $P < 0.01$) and tProDer f 1 (57.53 ± 5.12 μ g/ml, $P = 0.025$) (Fig. 6A). The IgG₁ levels were also significantly different for the R8 group (46.67 ± 7.89 μ g/ml) compared with the PBS group (0.14 ± 0.50 μ g/ml, $P < 0.01$), asthma group (96.11 ± 8.32 μ g/ml, $P < 0.01$), rProDer f 1 (74.15 ± 5.84 μ g/ml, $P < 0.01$) and tProDer f 1 (72.91 ± 5.52 μ g/ml, $P < 0.01$) (Fig. 6B). Finally, the level of IgG_{2a} in tR8 (76.20 ± 5.91 μ g/ml) was significantly higher compared to the PBS group (1.78 ± 0.68 μ g/ml, $P < 0.01$), asthma group (39.31 ± 4.59 μ g/ml, $P < 0.01$), rProDer f 1 group (63.79 ± 6.86 μ g/ml, $P < 0.01$) and tProDer f 1 group (66.31 ± 6.02 μ g/ml, $P < 0.01$) (Fig. 6C).

3.4. Pathological change of pulmonary tissue

Histologic examination of the lungs taken from mice in the asthma group showed intensive peribronchial and perivascular inflammatory infiltration, predominantly of eosinophils (Fig. 7B). Bronchial epithelial cells were overtly hypertrophied. Destruction of the smooth muscle layer, epithelial shedding, and goblet cells hyperplasia were also notable. In contrast, the negative controls exhibited normal lung architecture (Fig. 7A). Particularly, the respiratory epithelium was of normal size and morphology, and there was an absence of inflammatory cells in the peribronchial and perivascular regions. rProDer f 1-, tProDer f 1- and tR8- immunized mice also showed alleviated symptoms (Fig. 7C–E), compared with the asthma group.

4. Discussion

House dust mites of the *Dermatophagoides* species (*D. pteronyssinus* and *D. farinae*) are the important causative factor of various allergic diseases, such as bronchial asthma [42–44]. Group 1 (Der p 1 and Der f 1) allergens are considered to be one of the major allergens derived from house dust mites according to the frequency of patients sensitized by them, the amount of specific IgE produced against them, and their content in mite extract [45,46]. ASIT to mite allergy is routinely conducted using mite extracts prepared from natural allergen sources. These extracts hold numerous disadvantages that cannot be resolved with existing technical means [47], including undefined nonallergenic materials, difficulties on extract standardization, risk of inducing anaphylactic side-effects and induction of new IgE sensitizations, etc. [30,48].

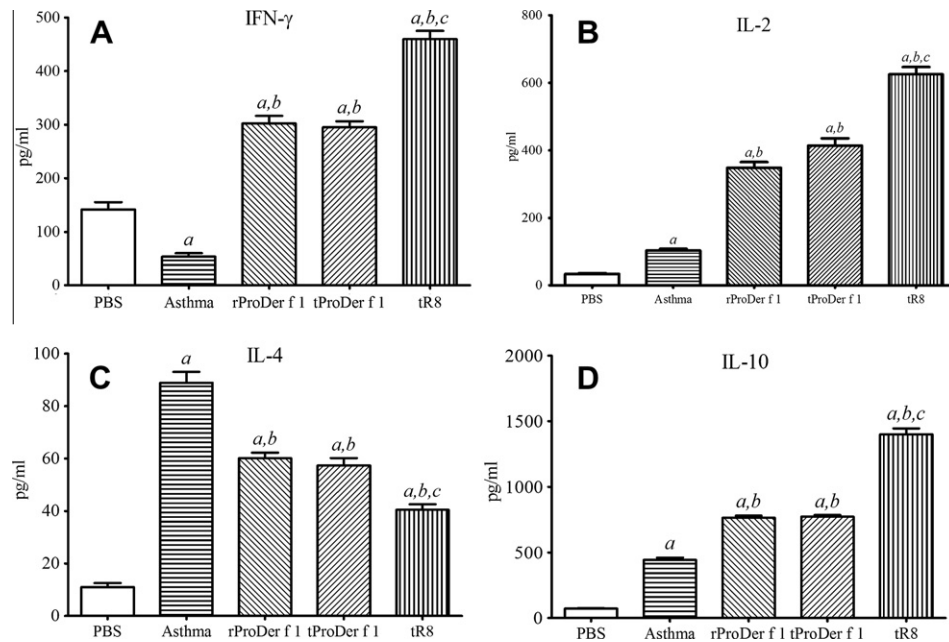


Fig. 5. The levels of IFN- γ , IL-2, IL-4 and IL-10 in BALF after ASIT. PBS as negative control, Asthma as control, rProDer f 1 as positive control 1 (ASIT with rProDer f 1 allergen purified from *E. coli*), tProDer f 1 as positive control 2 (ASIT with tProDer f 1 allergen purified from *N. benthamiana*), and tR8 as experimental (ASIT with tR8 allergen purified from *N. benthamiana*). Note: a, compared with PBS group, $P < 0.01$; b, compared with asthma group, $P < 0.01$; c, compared with rProDer f 1 and tProDer f 1 groups, $P < 0.01$.

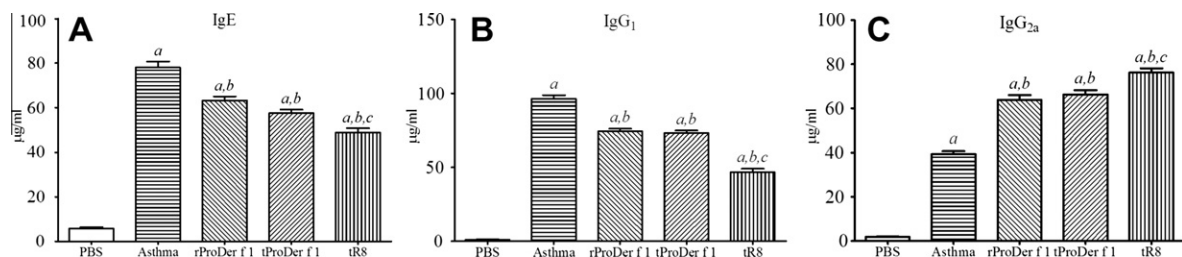


Fig. 6. The levels of serum IgE, IgG₁ and IgG_{2a} antibody in sera after ASIT. PBS as negative control, Asthma as control, rProDer f 1 as positive control 1 (ASIT with rProDer f 1 allergen purified from *E. coli*), tProDer f 1 as positive control 2 (ASIT with tProDer f 1 allergen purified from *N. benthamiana*), and tR8 as experimental (ASIT with tR8 allergen purified from *N. benthamiana*). Note: a, compared with PBS group, $P < 0.01$; b, compared with asthma group, $P < 0.01$; c, compared with rProDer f 1 and tProDer f 1 groups, $P < 0.01$.

Cloning and highly effective expression of recombinant allergens allows allergenic extracts to be replaced with expression systems such as *E. coli* and *Saccharomyces cerevisiae* [20,49,50]. Using plants as bioreactors is also considered a fine alternative to selecting highly purified allergens for ASIT vaccine production [5,51]. Previously, a chimeric gene R8, which was shuffled from two group 1 mite allergen genes, *ProDer f 1* and *ProDer p 1*, was screened successfully and encoded a protein characterized with hypoallergenicity and high T-cell immunogenicity [35]. In our study, we succeeded in producing recombinant R8 in a stable tobacco expression system. The chimeric molecule was detected by SDS-PAGE after agroinfection from the 3rd to 7th day, demonstrating a good stability of recombinant protein during plant development. The yield of R8 consistently rose from the 3rd day after agroinfection and achieved 4.54 ± 0.142 mg/g by the 7th day. Lindbo found the yield of GFP produced using the TRBO vector expression system in tobacco reached 5 mg/g [34]. These results confirm the usefulness of the TRBO vector, showing its remarkably high protein expression rate, which makes it easier to obtain useful quantities of target proteins. Specifically, our study shows that TRBO can produce R8 (for ASIT of asthma) with greater efficiency general agroinfection. Western blot analysis also suggests that R8 preserved

some epitopes of Der f 1. Furthermore, we observed a spontaneous processing of R8 forms in tobacco, which yielded mature R8 molecules. The mature R8 was probably formed through cleavage of pro-peptide by a tobacco protease. This finding was consistent with the observation of Burtin et al. [51] who found a mature form of the recombinant ProDer p 1 molecule expressed in tobacco.

Candidate recombinant allergy vaccines are mainly based on hypoallergenic mutants of natural allergens to improve immunogenicity and reduce allergenicity. For example, Burtin et al. [51] used biotechnology to successfully obtain four Der p 1 variants lacking N-glycosylation sites and/or cysteine protease activity. These variants exhibit characterization similar to natural Der p 1 with respect to IgE immunoreactivity. Chen et al. [50] synthesized two genes (rDer p 2/1C and rDer p 2/1S), which encoded hybrid proteins consisting of reassembled Der p 1 and Der p 2 fragments. rDer p 2/1C possesses cysteine activity, while rDer p 2/1S has no cysteine activity. Both rDer p 2/1C and rDer p 2/1S show lack of IgE-binding reactivity and inability to induce allergic inflammation after expression in *E. coli* and subsequent purification. In our study, we determined the binding capacity of R8 with IgE in sera from mite-allergic patients to further verify the effects of R8 expressed in tobacco on IgE-binding reactivity. Our results show that R8,

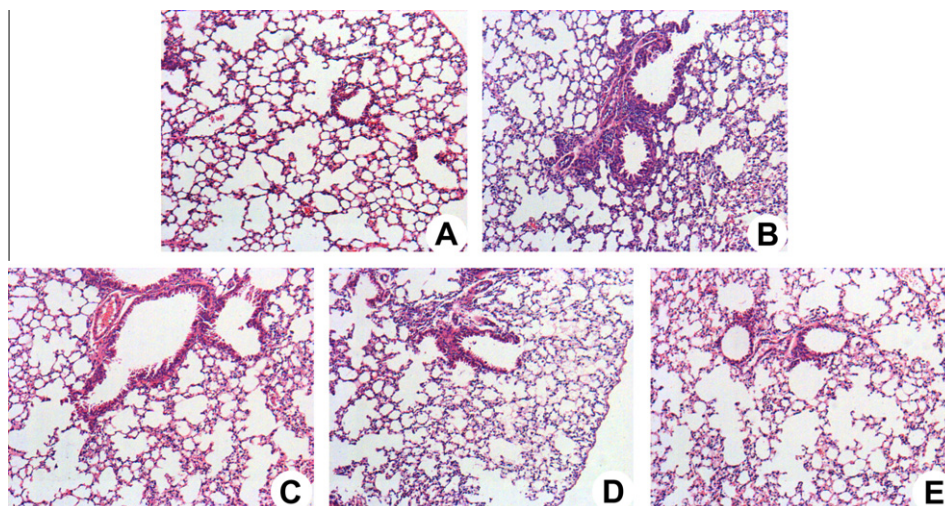


Fig. 7. Lung histopathology (HE staining, $\times 100$). The mice were sensitized and treated as described in the Materials and Methods section. Note the severe pulmonary inflammation in the areas adjacent to various sized airways in rProDer f 1-sensitized/challenged mouse (B), whereas the inflammation, goblet cell hyperplasia and mucus production were relatively minor in the lungs of mice treated with rProDer f 1, tProDer f 1 or tR8 (C–E).

rProDer f 1 and tProDer f 1 all have similar binding capacities with IgE, suggesting that R8 may bear properties identical to the parent allergen expressed in tobacco.

Successful ASIT leads to allergen non-responsiveness and symptom relief [52–54], including major changes in the T-cell response to allergens, either through immune deviation of T_H1/T_H2 imbalance or by induction of allergen-specific antibodies to prevent immediate type reactions. To further understand plant-expressed R8 for differentiation in subtypes of T_H cells, we determined the cytokine levels of IFN- γ , IL-2, IL-4 and IL-10 associated with T_H1/T_H2 /Treg cells using ELISA. As expected, the levels of IFN- γ and IL-2, cytokines predominantly secreted by T_H1 , were strikingly different from that of the parental protein expressed in *E. coli* or in tobacco. Moreover, the level of IL-10 showed a trend similar to that of IL-2. However, the IL-4 levels, primarily generated by T_H2 cell, were greatly reduced compared with the parent allergens. This result implies that R8 enabled activation of T_H1 - and Treg-cell differentiation and brought down the T_H2 cell growth concurrently, showing that R8 has properties similar to those of its parental proteins. Furthermore, the results of the IgG₁ and IgG_{2a} levels revealed that IgG₁ levels were significantly down-regulated in tR8 group but IgG_{2a} levels were strikingly raised compared with other experimental groups ($P < 0.01$). The findings suggest that tR8 has the potential to modify the imbalance of T_H1 and T_H2 cells and is favorable to T_H1 -biased allergic responses in agreement with the therapeutic principles for allergic disorders.

In conclusion, this study attempts to express a chimeric allergen R8 in tobacco for preserving or enhancing some characterizations of parental allergen. Observations of the experimental animals confirmed that the chimeric protein R8 is able to bear some properties of parental allergen, rProDer f 1 and tProDer f 1. Treatment with tR8 resulted in T_H1 cell predominance, suggesting that tR8 can competently rectify the T_H1/T_H2 imbalance.

6. Authors' contributions

Chao-pin Li devised of the study, participated in its design and coordination, and helped to draft the manuscript. Yu-xin Jiang carried out ELISA and wrote the manuscript. Wei Guo prepared the murine asthmatic model and performed statistical analysis. Zhi-ming Liu recruited the patients, collected blood samples, and performed SDS-PAGE. All authors examined and approved the final manuscript.

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