Construction of a *Der p* 2-Transgenic Plant for the Alleviation of Airway Inflammation

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Running title: Transgenic plant extracted protein inhibits asthma

Abstract

In clinical therapy, the amount of antigen administered in the application of oral tolerance for allergic diseases is large and the cost is a major consideration. To use tobacco plants for the development of a large-scale protein production system for allergen-specific immunotherapy and to investigate the mechanisms of transgenic plant derived antigen induced oral tolerance in this study. We used plants (tobacco leaves) transgenic for the *Dermatophagoides pteronyssinus* 2 (Der p2) antigen for the production of Der p2. Mice were fed with total protein extracted from Der p2 transgenic plants (TG plant) once per day over six days (days 0-2 and days 6-8). Mice were also sensitized and challenged with yeast-derived recombinant Der p2 (rDer p2), after which the mice were detected for airway hyperresponsiveness (AHR) and airway inflammation. After sensitization and challenge with rDer p2, mice fed with total protein extracted from TG plants showed decreases in serum Der p2-specific IgE and IgG1 titers, IL-5 and eotaxin levels in bronchial alveolar lavage fluid, and eosinophil infiltration in the airway. In addition, AHR was also decreased in mice fed with total protein extracted from TG plants and $CD4^+CD25^+Foxp3^+$ regulatory T cells were significantly increased in mediastinal and mesenteric lymph nodes in mice fed with total protein extracted from TG plants. Furthermore, isolated splenocytes from TG plant protein fed mice exhibited decreased proliferation and increased IL-10 secretion

after stimulation with rDer p2. The data here suggest that allergen-expressing transgenic plants could be used for therapeutic purposes for allergic diseases.

Key words: oral tolerance, asthma, transgenic plant, Der p2, IL-10

Abbreviation:

Der p: *Dermatophagoides pteronyssinus*

AHR: airway hyperresponsiveness

TG: transgenic

i.t.: intratracheal

i.p.: intraperitoneal

Introduction

Asthma affects nearly 155 million individuals worldwide, and the incidence is still increasing[1]. The dysregulation of allergen-specific immune responses plays a central role in the generation of asthma. Clinical and experimental studies of allergic inflammation/asthma suggest $CD4^+$ T helper (Th) 2 cells as the major effector cells in airway inflammation, being associated with lung dysfunction by the recruitment and activation of eosinophils [2-4].

Oral tolerance, or the oral administration of antigen which induces specific inhibition of cellular and humoral immune responses, has been applied in numerous disease therapeutic studies. For example, the administration of oral antigen suppresses animal models of autoimmune diseases including experimental autoimmune encephalitis and arthritis, as well as non-autoimmune diseases such as asthma, allergy, stroke, and Alzheimer's diseases[5]. Several mechanisms of oral tolerance have been proposed: deletion of antigen-specific T cells (clonal deletion)[6], immune deviation[7], induction of anergy[8], and suppression of antigen-reactive cells by regulatory T cells (Tregs)[9].

In clinical settings, oral tolerance has been applied to treatment of allergic diseases. For example, specific immunotherapy (SIT) has been practiced on patients in an attempt to achieve tolerance by the administration of low dose allergen over long

periods of time via different routes including subcutaneous, sublingual, oral, local bronchial, and local nasal delivery [10]. The traditional subcutaneous route is burdened with the risk of severe adverse events; therefore, safer routes of administration such as the sublingual route, is supported by numerous controlled trials showing its long-lasting efficacy in asthma and rhinitis in adults and children [11-14]. Sublingual immunotherapy is now accepted by the World Health Organization as a valid alternative to the subcutaneous route in children asthmatics [15, 16].

However, sublingual immunotherapy still has its drawbacks in that the duration of allergen administration is long and the cost is considerable[11]. Developing a convenient and cost-effective delivery method is still required. Allergen-transgenic plants can serve this purpose since the cost of large-scale production is very low and extraction procedures may not be needed (using edible plants directly). Also, the exceptional stability of transgenic proteins allows for ease of storage and transportation[17]. In addition, using plants to express recombinant proteins also prevent unwanted byproducts such as endotoxin in an E coli expression system.

Here we have proven that feeding transgenic plant-derived allergen protein to a murine model of asthma could suppress airway inflammation and hyperresponsiveness. This study further indicates that oral delivery of allergen proteins extracted from transgenic plant may be a feasible approach for the treatment

of allergic diseases.

Materials and Methods

Generation of Der p2-transgenic plant and recombinant Der p2

Transfection was done by co-culture of 100μl of Der p2-pCambia2300 (CAMBIA, Canberra, Australia) plasmid transformed *Agrobacterium*-containing MSG medium (MS salts, Gamborg's B5 vitamins, 3% sucrose, pH5.8) plus 0.5 ml 200μM acetosyringone with 3-week old tobacco (*Nicotiana tabacum* L. cv. W38 strian) 1cm×1cm leaves slices onto BM agar plate (MS salts, Gamborg's B5 vitamins, 0.4 μg/ml BAP, 3% sucrose, 0.8% agar, pH5.7) for two days in the dark at 27^0C.

Infected leaves slices were transferred to 200 μg/ml Kanamycin and 300 μg/ml cefotaxime-HCl– containing BM agar plates in 16L/8D light at 27^0C. The leaves germinated within 3-4 weeks. Transfected *Agrobacterium* clones were checked by PCR. To measure the Der p2 expression level of each clone, we performed Western blotting using the anti-Der p2 monoclonal antibody kindly provided by Dr. KT Lee's lab at the Institution of Agriculture Chemistry, NTU .

Plant cell suspension cultures and protein extraction was accomplished by Dr. KT Lee's lab at the Institution of Agriculture Chemistry, NTU. Briefly, callus from a positive clone named +11 (which expressed Der p2 in the cytosol at 0.5% of total protein) was added to the suspension culture without light. Total protein was extracted

and concentrated by ultra filtration which excluded low molecular weight molecules, such as nicotine and tobacco tar. The total protein was frozen in liquid nitrogen then preserved in -20^{0}C. Each vial was thawed only once, and preserved in 4^{0}C after being centrifuged at 12,000 rpm to discard aggregations. Der p2 protein concentrations were quantified by western blot using recombinant Der p2 as a standard.

Recombinant Der p2 was generated by *pichia* (His$^+$ Mut^s), which was kindly provided by Dr. KY Chua (Department of Paediatrics, National University of Singapore). For 1 liter of BMGY medium culture ($O.D._{600}$ = 6), induction was performed in 200 ml BMMY medium for 48hrs at 30^{0}C. Culture supernatant was collected and dialyzed against 20mM CHCOONa, pH=5.0 (Binding buffer). Then the sample was applied to HighTrap SP Sepharose (Pharmacia) for ion-exchanging purification. Protein samples, binding buffer, wash buffer containing 10mM NaCl and elute buffer containing 150mM NaCl were all 0.22μm filtered before use.

Establishment of murine model of asthma

Female BALB/c mice 6-8 weeks old were obtained from the Animal Center of the College of Medicine. National Taiwan University Animal Care and Handling protocols were approved by the Animal Committee of National Taiwan University. The BALB/c mice were immunized with intraperitoneal injections of 30 μg of rDer p2 mixed with 4 mg alum (Pierce, Rockford, IL, USA) plus 200 ng pertussis toxin

(List Biological Lab, Cambell, CA, USA) on days 1, 14, and 28. On days 1-3 and 6-8, mice were fed with 651μg protein extracted from transgenic plant containing 100μg recombined Der p2 (rDer p2), 651μg protein extracted from wild type plant, 100μg rDer p2 extracted from yeast, or buffer, once per day. All proteins were dissolved in 3% Na_2CO_3 buffer. On day 21, all mice were bled to measure the Der p2-specific IgG and IgE titers by sandwich ELISA. On day 34 and 36, all mice were challenged by intratracheal injections of 5μg of recombinant Der p2 antigen to induce airway inflammation. The negative control group represented mice that were not immunized with Der p2.

Bronchoalveolar lavage and lung histology

Bronchoalveolar lavages (BAL) were performed using 1ml HBSS, instilled bilaterally with a syringe. The bronchoalveolar lavage fluid (BALF) was harvested by gentle aspiration three times and then centrifuged. Differential cell counts were assessed on cytologic preparations. The cell slides were prepared with use of a cytospin and stained with Liu staining. A total of 300 cells were counted using microscopy. BAL fluid supernatants were assayed afterward by ELISA.

The lungs were fixed with 10% neutral phosphate-buffered formalin. Sections were prepared and stained with hematoxylin/eosin (H&E) to quantitate the number of infiltrating inflammatory cells under microscopy.

Determination of cytokine expressions

IL-4, IL-5, IL-10, TGF-β, eotaxin, IL-13, and IFN-γ levels were measured by the ELISA method according to the procedures recommended by the manufacturer (R&D, Minneapolis, MN, USA.).

Splenocytes proliferation assay

Cells were cultured in AIM-V medium supplemented with 2% TCM (mouse serum replacement, Celox Corp., Oakdale, MN, USA) in the presence or absence of recombinant Der p2 (5, 10, 20 μg/ml) or anti-CD3 plus anti-CD28 (1μg/ml each). After incubation for 2 days, 1μCi/well of [H^3]TdR was added and incubated for 17 h. Cpm (counts per minute) values were read with a β-counter (Packard Instrument Co., Meriden, CT, USA). The SI (stimulation index) was defined as the cpm of stimulated cultures divided by the cpm of cultures in media only.

Whole body plethysmography

Airway responsiveness was measured in unrestrained animals by barometric whole body plethysmography (Buxco, Troy, NY. U.S.A.) [18]. Briefly, mice were placed in the main chamber, and baseline readings were taken and averaged for 3 min. Aerosolized PBS or methacholine (MCh) in increasing concentrations (6.25 to 50 mg/ml) were nebulized through an inlet of the main chamber for 3 min, and readings were taken and averaged for 3 min after each nebulization. Recordings of every 10 breaths were

extrapolated to define the respiratory rate in breaths per minute. Airway reactivity was expressed as an enhanced pause (Penh), and data were expressed as the ratio of $Penh_{MCh}$ values compared to $Penh_{PBS}$ from three independent experiments.

Measurement of airway resistance in anesthetized mice

Airway resistance was assessed as an increase in pulmonary resistance after challenge with aerosolized MCh in anesthetized mice using a modification of the techniques described by Glaab et al. [19]. Mice were anesthetized with 70-90 mg/kg pentobarbital sodium (Sigma), tracheostomized, and mechanically ventilated at a rate of 150 breaths/min, a tidal volume of 0.3ml, and a positive end-expiratory pressure of 3-4 cmH_2O with a computer-controlled small animal ventilator (Harvard Rodent Ventilator, model 683, Southnatick, MA, USA.). PE-50 tubing was inserted into the esophagus to the level of the thorax, coupled with a pressure transducer (LDS GOULD, Valley View, OH, USA.). Flow was measured by electronic differentiation of volume signal. Pressure, flow, and volume changes were recorded. Pulmonary resistance was calculated by the software program (Model PNM-PCT100W, LDS PONEMAH Physiology Platform, LDS GOULD). 25 mg/ml MCh aerosol was generated with an in-line nebulizer and administrated directly through the ventilator. The resistance of the orotracheal tube ($0.48cmH_2O.s.ml^{-1}$) was subtracted from all airway resistance measurements.

Der p2-specific antibody assay

ELISA determined total sera and anti-Der p2 immunoglobulin (Ig) E and IgG1 antibody titers. Briefly, 96-well microtiter plates were coated with 10 μg well^{-1} Der p2 (Pharmingen, San Diego, CA, USA) in $NaHCO_3$ buffer (pH 9.6). After overnight incubation at 4°C, the plates were washed and blocked with 3% bovine serum albumin (BSA) in PBS for 2 h at room temperature. Serum samples were diluted and added to each well overnight at 4°C. Plates were then washed. Either biotin-conjugated anti-mouse IgE or IgG1 (0.5 mg mL^{-1}, Pharmingen) diluted in 3% BSA-PBS buffer (1:500) was added for 45 min at room temperature. Avidin-conjugated horseradish peroxidase (1:5000, Pierce Biotechnology, Rockford, IL, USA) was then added for another 30 min at room temperature. The reaction was developed by peroxidase substrate, 3,3′,5,5′-tetramethylbenzidine (KPL, Gaithersburg, MD, USA) and then stopped by 2N H_2SO_4. Absorbance was determined at 450 nm in a microplate reader. Antibody levels were compared to standard serum, and IgG1 and IgE concentrations in standard serum were arbitrarily assigned 1 ELISA unit (1 EU).

Lymph node preparation

Mediastinal and mesenteric lymph nodes were harvested and pooled from each group at time of sacrifice. Single-cell suspensions were obtained by mechanical disruption.

Cells were stimulated *in vitro* with 5 µg/mL Der p2 for 72 h. The cell medium was collected for cytokine analysis.

Flow cytometry analysis

Freshly isolated lymph nodes were incubated with FITC- and allophycocyanin-labeled mAbs to mouse CD4 and CD25 (eBiosciences, San Diego, CA, USA). For analysis of intracellular Foxp3, stained cells were fixed and permeabilized with Cytofix/Cytoperm solution (ebioscience) according to the manufacturer's suggested protocol and then incubated with PE-conjugated anti-Foxp3 (eBiosciences) o at 4°C for 30 min in the dark. Cells were then washed and analyzed by flow cytometry (FACSCalibur; BD Biosciences).

Statistical analysis

All values refer to mean ± S.E.M. using one-way ANOVA followed by Newman-keuls post-hoc comparisons. The criterion significance was set at p-values < 0.05.

Results

Generation of transgenic plants

We used the pCambia2300 vector that contained transferred DNA (T-DNA) for the expression of Der p2. These Der p2 expressing pCambia2300 vectors were then transferred into tobacco leaf slices by *Agrobacterium*-mediated transformation.

To get the optimal expression level among cellular compartments, three different plasmid constructs that target protein secretion to different organelles were constructed and summarized in Figure 1a. One containing an enhancer (E) sequence for translation in the N'-terminus of the target gene was derived from Jobling and Gehrke, 1987 [20], which would express the protein product in the cytosol. The other two constructs containing signal peptides (SP) would go through a secretory pathway and excise the SP after localization. Among them, the one without a stop signal would transport the protein product out of the cell membrane. The other has a vacuolar targeting signal (NTPP) that would transport the protein product to the vacuole [21], the largest organelle in plant cells. Soramin is a protein accumulated in large quantities in vacuoles, so speculation has been made that larger spaces might be important to enhance protein secretion. That is why we wanted to target our protein to the cellular matrix or vacuole, both of which are larger areas than the cytosol.

The highest expression level of Der p2 protein obtained was 0.5% of total protein (Figure 1b) in the cytosol-targeting group, while the group with the highest frequency of Der p2 positive clones by western blotting was in the vacuole-targeting group. However, the overall expression levels were similar between the cytosol-targeting group and vacuole-targeting group (Figure 1b). The ratio of the target protein to total protein in our system was higher than that obtained from a previous study [17]. In this study, we chose the clone with the highest Der p2 expression level (Der p2 expressed in the cytosol) for the plant cells protein extraction. The amount of Der p2 from a total protein extraction of 35 g dry weight callus can be up to 0.185g at the maximum. The Der p2 expression in transgenic tobacco plant cells are shown in Figure 1c and after extraction and concentration, the Der p2 expression level elevated to about 15% of total protein.

Feeding total protein extracted from Der p2 transgenic plants to sensitized mice suppressed Der p2-specific IgE and IgG1 titers.

The *in vivo* effect of Der p2 feeding was analyzed by feeding BALB/c mice with Der p2 from different sources. First, we investigated whether oral feeding of total protein extracted from Der p2 transgenic plants (TG plant) could decrease serum Der p2-specific IgE and IgG1 titers.

Mice were fed with 0.1mg per day of yeast-derived rDer p2 or total protein

extracts from TG plant containing 0.1mg Der p2 starting on the day of immunization for three days (day 0, 1, 2) and another three days in the following week (day 6, 7, 8). We orally fed the mice time points when the immune response to Der p2 was not established (Figure 2a). Seven days after the boost, serum levels of Der p2-specific antibodies were detected in these mice. Both Der p2-specific IgE and IgG1 titers decreased after feeding (Figure 2b). In addition, the inhibition of Der p2-specific IgE titer was sustained after Der p2 challenge (Figure 2c).

Feeding mice with total protein extract from Der p2 transgenic plant suppressed airway inflammation and BALF cytokines release

The Der p2-sensitized mice were subsequently challenged via i.t. using recombinant Der p2 (5μg on day 34 and 36) to induce airway inflammation. Feeding with total protein extracted from Der p2 transgenic plants (oral TG plant group) or recombined Der p2 (rDer p2) extracted from yeast (oral rDer p2 group) to mice appeared to inhibit the recruitment of eosinophils to the airway in BALF, as compared with mice orally fed with total protein extracted from wild type plants (oral WT plant group) or buffer (oral buffer group) (Figure 3a). Histological studies also support this conclusion as shown in Figure 3b. Lung tissue from oral WT plant and oral buffer groups showed numerous inflammatory cells surrounding the airways and streaks of mucus in the lumen. After oral feeding with total protein extracted from TG plants or

rDer p2 from yeast, Der p2-sensitized and challenged mice showed minimal mucus production and negligible cellular infiltration as in negative control mice that were not immunized with Der p2.

Further investigation of BALF cytokine expression after oral feeding of total protein extracted from TG plants or rDer p2 from yeast revealed decreased IL-4, IL-13, IL-5, and eotaxin levels as compared with the oral WT plant and oral buffer groups (Figure 3c).

Airway hyperresponsiveness decreased after oral feeding with Dp2 transgenic plant

Furthermore, we analyzed whether oral feeding of total protein extracted from TG plant would affect the development of airway hyperresponsiveness (AHR) in a murine model of asthma. One day after the final challenge, airway responsiveness was assessed by non-invasive whole body plethysmography and pulmonary resistance using invasive body plethysmography. BALB/c mice that had been sensitized and challenged with Der p2 revealed an increase in the Penh ratio (Figure 4a) and lung resistance (R_L) (Figure 4b) to methacholine (MCh) inhalation over PBS-sensitized and challenged mice (Figure 4, the oral WT plant and oral buffer groups versus the negative control group). After oral feeding of total protein extracted from TG plants and rDer p2 from yeast, the levels of Penh ratios and R_L were similar to those of the negative control group; therefore, Der p2-sensitization and challenge induced AHR

formation was suppressed in mice fed with TG plant Der p2 or rDer p2, as compared with the oral buffer or oral WT plant groups.

Oral feeding of Dp2 transgenic plant increases $CD4^+CD25^+Foxp3^+$ regulatory T cells in mesenteric and mediastinal lymph nodes

We found that oral feeding of total protein extracted from TG plants induced mucosal tolerance as rDer p2. Next, we examined the mechanisms of oral feeding of total protein extracted from TG plant induced mucosal tolerance. On day 38, we identified $CD4^+CD25^+Foxp3^+$ regulatory T cells from mediastinal (LLN) and mesenteric lymph nodes (MLN). After oral feeding of total protein extracted from TG plants, the level of $CD4^+CD25^+Foxp3^+$ regulatory T cells in MLN and LLN was significantly increased compared to mice with oral fed buffer or total protein extracted from WT plant (Fig 5a). Next, we detected cytokines expression in MLN cells. After stimulation with rDer p2, the level of IL-4 was significantly decreased in mice oral feeding of total protein extracted from TG plants, as compared to mice with oral fed buffer or total protein extracted from WT plant (Fig 5b). We did not detect the IFN-γ expression among different groups.

Decreased proliferation response and increased IL-10 secretion in splenocytes from mice fed with transgenic plant upon antigen stimulation

To further investigate the systemic immune response after oral feeding of total

protein extracted from TG plants, we cultured splenocytes from Der p2-sensitized and challenged mice fed with proteins derived from TG or WT plants. At the stimulation of rDer p2, the stimulatory index (S.I.) value of the oral TG fed group was significantly lower than that of the wild type plant group (Figure 6a). IL-10 in the supernatants of the oral TG group was significantly higher than that of the oral WT plant group (Figure 6b).

Discussion

Dermatophagoides pteronyssinus is a major allergen worldwide. Approximately 80% of asthmatics in Taiwan are sensitized by the house dust mite *Dermatophagoides pteronyssinus* (*Der p)*. Among them, 87.8% (85.4% of adults and 90.2% of children) of the asthmatic patients in Taiwan with Der p hypersensitivity were allergic to Der p2[22]. However, most of the information about mucosal tolerance as treatment for allergic diseases comes from murine models using ovalbumin (OVA) as the model antigen. OVA is an airway inflammation-inducing allergen used in many murine models because of its availability, but most patients are not allergic to eggs[9]. Therefore, in this study, we used Der p2 as an antigen to establish a murine model of asthma. Orally feeding mice with total protein extracted from TG plant effectively inhibited Der p2-specific IgE and IgG1 titers, BALF IL-4, IL-5, IL-13, and eotaxin production, airway inflammation, airway hyperresponsiveness, suppressed spenocytes proliferation.

Specific immunotherapy with whole allergen could induce unwanted anaphylactic reactions in patients, as indicated by increasing serum antigen-specific IgE levels during the desensitization process[23, 24]; however, this phenomenon was not observed in our study and oral feeding with total protein extracted from TG plants in fact suppressed the Der p2-specific IgE level in serum. In addition, a previous study found

that long term subcutaneous specific immunotherapy with native allergen Der p 1 and Der p2 decreased serum specific IgE and asthma symptoms in patients[25]. Therefore, oral feeding with whole allergen is also feasible with regards to specific immunotherapy for asthma.

The mechanisms of oral tolerance induced by the feeding of total proteins extracted from TG plants were investigated by performing the following experiments. Recently, many researchers found orally induced Treg cells are functional and inhibit inflammation both locally in the gut and systemically. For example, oral fed ovalbumin to induce adaptive $Foxp3^+$ Treg cells which inhibited allergic inflammation in mice[26]. Therefore, first, we examined the expression of regulatory T cells (Treg) in mediastinal (LLN) and mesenteric lymph nodes (MLN) and we found that the expression of regulatory cells in LLN and MLN was increased in mice fed with total protein extracted from TG plants. The mechanisms of Treg cells- induced inhibition of effector T cells can be through inhibitory cytokines such as IL-10 or TGF-β , cytolysis, metabolic disruption (cAMP-mediated inhibition, CD25-mediated apoptosis, and CD39- and/or CD73-generated, adenosine receptor 2A-mediated immunosuppression, and inhibition dendritic cell maturation and function[27]. We also investigated the suppressive cytokines by measuring IL-10 and TGF-β in BALF and splenocytes culture supernatants. These cytokines were not detected in BALF; however, IL-10

levels in splenocyte culture supernatants were increased in mice fed with total protein extracted from TG plants. IL-10 has been thought to be involved in several regulatory mechanisms including the down-regulation of mast cell function, suppression of IgE, decreased eosinophil-sensitive chemokine production and reduced eosinophil survival[28]. These results support the notion that suppression of experimental allergy by transgenic plants may be associated with the induction of the role of IL-10. Recently, expression of IL10 is not specific for Th2 or Treg but instead that it is a much more broadly expressed cytokine. IL-10 can expressed in immune cells of adaptive immune system such as Th1, Th2, Treg, CD8+ T cells and B cells[29]and innate cells such as dendritic cell, macrophage, natural killer cells, mast cells, eosinophils, and neutrophils[30]. Therefore, any significant contribution of IL-10 in DP2 transgenic plant-mediated suppression of allergic airway disease would require further investigation.

Secondly, we measured the content of a Th1 cytokine, IFN–γ, in the BALF of mice that is known to exert inhibitory effects on allergic responses[31]. We found that after Der p2 immunization, BALF, MLN, and splenocyte culture supernatant IFN-γ levels from mice fed with the transgenic plant were below detection level (data not shown). We also found that the serum specific IgG2a levels were also low in mice fed with total protein extracted from the Dp2 transgenic plant**,** similar to those found in mice

fed with total protein extracted from wild type plants (data not shown). Thus, IFN-γ appears not to be involved in the TG induced oral tolerance in murine model of asthma.

In addition, we also found that decreased lung inflammation from mice with oral feeding of total protein extracted from WT plant in lung tissue section. We thought that it might be associated with increasing the Treg cells in mediastial lymph nodes; however, the mechanism needs further investigation.

The development of plant-derived pharmaceuticals for disease therapy is an emerging field in vaccine research [32], especially since models using viral and bacterial infections may have unwanted side-effects. Current conventional specific immunotherapy is expensive to prepare, and require multiple infections of allergen over a period of years[33]. Thus, the availability of proper allergens expressed in transgenic plants in large quantities may facilitate further clinical therapy for the establishment of suppressive responses in allergic diseases. According to previous studies [34], one of the limitations in the development of transgenic plants for pharmaceuticals is the low expression level of recombinant foreign protein. In general, levels of recombinant protein produced by transgenic plants range from 0.001-1% of total soluble proteins and may not compete with other recombinant protein production system (E coli or yeast) at present. However, the advantages of plant systems such as

the potentially high accumulation levels of the protein, posttranslational processing, and natural storage stability are important factors for expressing transgenic proteins in plant[35]. The cost of downstream processing may determine whether a particular plant production is competitive with traditional fermentation systems. Using partially purified plant proteins or eatable plants, which do not need purification, can provide low production costs of recombinant proteins. Thus, an important project currently underway is the generation and examination of the Der p2 transgenic tomato plant.

In this study, the expression levels of Der p2 in the transgenic tobacco plants of this study reached up to 15% of the total extracted protein. This higher protein purity from the extraction system used in this study can induce oral tolerance in allergic asthma and demonstrate therapeutic potential for other allergic diseases. In conclusion, the remarkable potential therapeutic effects of the Der p2-transgenic plant in our study implicate that in the future, Der p2-transgenic plants may be a desirable choice of treatment for allergic patients.

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Figure 1. Transfer-DNA (T-DNA) regions of plasmids constructs used to create the transgenic plant. (**a**) T-DNA regions of plasmids constructs used to create the transgenic plant. (**b**) The structure of T-DNA regions and results of plant clones screening of the three transgene constructs. (**c**) Der p2 expression of protein extracted from transgenic plants by western blotting analysis. Signal peptide (SP), endoplasmic reticulum (ER), ER retension signal (HDEL), Vacuole targeting signal (NTPP), CaMV35S promoter (CaMV P).

Figure 2. Detection of specific antibodies after sensitization of mice fed with Der p2 protein. (**a**) Time course of Der p2 oral feeding and asthma murine model setup. (**b**) Mice fed with either recombinant Der p2 or Der p2-transgenic plant extract expressed diminished Der p2-specific IgE and IgG1 titers when mice were bled a week after the first boost. (**c**) Der p2-specific IgE titers when mice were bled one day after final challenge. NC represents a negative control group where the mice were not immunized with Der p2. Data were expressed as mean ± S.E.M. ($n \geqq 6$). *$p < 0.05$, **$p<0.01$, ***$p<0.001$.

Figure 3. Decrease of airway inflammation and BALF cytokines after feeding with total protein extracted from Der p2-TG plant in mice. (**a**) Inflammatory cell profile in BALF. (**b**) Histopathological examination of lung tissues. (**c**) Cytokines levels in BALF. Data are expressed as mean ± S.E.M. NC

represents negative control group. (n≧6). *p < 0.05 **p<0.01, ***p<0.001.

Figure 4. Airway hyperresponsiveness inhibited by oral feeding of total protein extracted from Der p2-TG plants in mice. (**a**) Airway hyperresponsiveness was measured by non-invasive whole body plethysmography as described in the Methods section. Data were expressed as the mean ± SEM of the Penh values in the ratio of Penh values after PBS nebulization of three independent experiments (n≧5). NC represents the negative control group. a*p<0.05, a**p<0.01, a***p<0.001 versus negative control group. b***p<0.001 versus oral WT plant group. c***p<0.001 versus oral buffer group. (**b**) Airway resistance as measured by invasive body plethysmography. Data were expressed as the mean ± S.E.M. of the pulmonary resistance (RL) in the ratio of RL after PBS nebulization of three independent experiments (n≧5). ***p<0.001. NC represents negative control group.

Figure 5. Increase in frequency of $CD4^+CD25^+Foxp3^+$ cells in mesenteric (MLN) and mediastinal lymph node (LLN) and decrease in IL-4 production in mesenteric lymph node cells after oral feeding of total protein extracted from Der p2-TG plant. (a) On day 38, the frequency of $CD4^+CD25^+Foxp3^+$ cells in MLN and LLN were analyzed by flow cytometry. (b) On day 38, isolated MLN cells treated with 5μg/ml rDerp2 for 72h and supernatants were

collected for analysis of cytokines production by ELISA. Data are expressed as mean ± S.E.M. (n≧3) NC represents negative control group. #p<0.001 compared with NC group, *p < 0.05 **p<0.01, ***p<0.001.

Figure 6. Decreased proliferation and increased IL-10 production in splenocytes from mice fed with total protein from Der p2-TG plant. (**a**) Proliferation of splenocytes upon stimulation with rDer p2. Mice were fed transgenic plant extracts containing 100μg Der p2 or wild type plant extract from the day of immunization. (**b**) IL-10 secreted in the splenocytes supernatants of splenocyte cultures upon rDp2 stimulation. NC represents the negative control group. Data were expressed as mean ± S.E.M. (n≧5).*p < 0.05, ***p<0.001 versus the oral WT plant group.