Oral immunogenicity of potato-derived antigens to Mycobacterium tuberculosis in mice

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The novel use of transgenic plants as vectors for the expression of viral and bacterial antigens has been increasingly tested as an alternative methodology for the production and delivery of experimental oral vaccines. Here, we examined the immunogenicity of combined plant-made vaccines that include four genes encoding immune-dominant antigens from Mycobacterium tuberculosis. Compared with the wild type and other control groups, mice treated with the combined plant-made vaccines showed significantly higher levels of interferon-γ and interleukin-2 production in response to all four proteins, and higher levels of antigen-specific CD4+ and CD8+ T-cell responses and immunoglobulin (Ig) G and IgA titers. These results suggest that combined plant-made vaccines can induce immunogenicity against M. tuberculosis through the induction of stronger Th1-associated immune responses. This is the first report of an orally delivered combined plant-made vaccine against tuberculosis priming an antigen-specific Th1 response, a comprehensive effect including both mucosal and systemic immune responses.

Keywords tuberculosis vaccine; mucosal delivery; transgenic plants

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Introduction

Mucosal-administered vaccines have the potential to induce humoral and cell-mediated immune responses at mucosal sites and at the systemic level [1]. This attribute, together with a needle-less, non-invasive approach, makes mucosal vaccines very attractive. Plant-based edible vaccines have the advantages of mucosal vaccines, along with other attractive attributes, including the lack of fermentation and protein purification, cost-effective production (due to low-energy input), low supply costs, easy transportation, preservation, and delivery [2]. Plant cell walls and membranes provide ‘bioencapsulation’ to protect antigens from degradation by digestive enzymes to some degree within the gastrointestinal track [3], so a better immune response can be obtained by the oral delivery of raw materials expressing the desired antigen [4]. Plants have the ability to generate pathogenic bacterial antigens that maintain their biological functions. Moreover, edible vaccines could be particularly suitable for farm animals destined for meat, because the alternative vaccination route of repeated injections can cause deterioration in carcass quality [5,6].

Tuberculosis (TB), caused by the respiratory pathogen Mycobacterium tuberculosis, remains a major global health problem, killing about 2 million individuals each year. It was reported that there were about 8.8 million incident cases of TB, 1.1 million deaths from TB among human immunodeficiency virus (HIV)-negative people, and an additional 0.35 million deaths from HIV-associated TB all over the world in 2010 [7]. And actually, the incidence rate has not decreased these years in both developing and industrial countries [8]. Recent studies have indicated that a major proportion of the global TB burden occurs in developing countries, where the incidence of TB is accelerated by immunocompromisation caused by HIV infection and poverty [9]. The current Bacille Calmette–Guerin (BCG) anti-TB vaccine, which consists of attenuated Mycobacterium bovis, is administered annually to over 100 million children worldwide [10]. It is effective in infants but fails to protect adolescent or adults against pulmonary TB, and is detrimental to some immunodeficient individuals [11]. One new approach is to develop more effective vaccines against TB. The use of transgenic plants for the expression of recombinant antigens has been increasingly used for the production of experimental immunogens [12–15]. The respiratory track is the portal of entry and primary site of infection of M. tuberculosis, and therefore mucosal vaccination should have an inherent advantage in inducing a protective immune response. Unfortunately, parenteral immunization, the traditional means of vaccine delivery, is a poor inducer of mucosal immunity. Transgenic plants expressing therapeutic proteins may be ideal vehicles to produce and orally deliver...
protective antigens. The plant cell wall may delay digestion of plant-made antigens [15–18], resulting in more antigen being taken up and presented to gut-associated lymphoid tissue.

The major antigens produced by *M. tuberculosis* during infections are antigen 85B (Ag85B), a 31-kDa mycolyl transferase involved in cell wall biogenesis, and early secreted antigenic target-6 (ESAT6), a small 6-kDa protein, possibly involved in immune modulation [19–22]. Other antigens are MPT64, which is secreted by a number of strains of mycobacterium and recognized by the immune systems of the majority of TB patients and their contacts, and MPT83, a lipitated and glycosylated protein that remains attached to the cell surface to facilitate the exposure of epitopes. MPT83 is recognized by T lymphocytes from infected individuals [23,24]. The Ag85B, MPT64, MPT83, and ESAT6 antigens are all dominant targets for cell-mediated immunity and are capable of inducing strong immune responses in a number of animal models [19–24]. The fusion of Ag85B to ESAT6 (Ag85B–ESAT6) is more immunogenic and gives higher levels of protection compared with the individual antigens, when administered parenterally [25,26]. In addition, intranasal immunization regimens with Ag85B-ESAT6 have shown promise [25]. The function of the secreted protein MPT64 is unknown, but it is expressed only in actively dividing cells; Huang *et al.* [26] have evaluated the potential of SLDAPD to deliver DNA vaccine vectors carrying the *M. tuberculosis* antigen MPT64. The combination of Ag85B, MPT64, and MPT83 has produced a higher protective immune response than vaccines containing the individual components alone [27]. A plant-made LTB–ESAT6 fusion protein induced antigen-specific responses in CD4+ cells and increased interferon-γ (IFN-γ) production, indicating a Th1 response [6]. However, the efficacy of oral vaccines containing genes encoding MPT83, Ag85B, MPT64, and ESAT6 against *M. tuberculosis* has not been reported. Here, we investigated the ability of transgenic plants to serve a novel delivery system for an oral immunogenic TB vaccine in mouse feed, and to find an effective combination of multiple antigens. The expressed products were shown to be immunogenic in an experimental mouse model by inducing a strong antigen-specific antibody response, as well as a Th1 cellular immune response.

### Materials and Methods

#### Construction of plant expression vectors

The plasmid named CAMBIA 2300-Patation was used for final tuber-specific expression. It was made by cloning the *Hindl–EcoRI* fragment from pCAMBIA 1305, containing CamV35S promoter and Nos terminator into pCAMBIA 2300, and both plasmids were kindly provided by Prof. Zhong-Ping Lin of Peking University (Beijing, China). Then the tuber-specific class I patatin promoter (gift of Prof. Zhong-Ping Lin), which controls the high-level expression of a class of proteins in tuber, was subsequently inserted into the *HindIII/BamHI*-digested plasmid by replacing the CamV35S promoter [Fig. 1(A)]. The coding regions for antigens Ag85B (AY207396.1), MPT83 (X94597.1), MPT64 (AY208674.1), and ESAT6 (AF420491.1) were amplified from *M. tuberculosis* H37Rv chromosomal DNA using designed primers based on the corresponding genomic sequence database. The primers for each protein from the 5’ to the 3’ end, and including a *SacI/BamHI* enzyme site, were: Ag85B sense, TACGGATCCATGACAGCGTGAGCCGAAAGATT; Ag85B antisense, GATGAGCTCCTTAAGTTTGGTCGCTTTTGCCCGGCCCTAAAGGA; MPT83 sense, GAGTTCGTCTTTGCACCGGCCCTAAAGGA; MPT83 antisense, GAGCAGCTCAGGAGGCACGTCTACAG; MPT64 sense, GAGTTCGTCTTTGCACCGGCCCTAAAGGA; MPT64 antisense, GAGCAGCTCAGGAGGCACGTCTACAG; ESAT6 sense, GAGTTCGTCTTTGCACCGGCCCTAAAGGA; ESAT6 antisense, GAGCAGCTCAGGAGGCACGTCTACAG.

The polymerase chain reaction (PCR) product was digested with *SacI* and *BamHI* before ligation into the plant expression vector pCambia2300-patatin and subsequently transformed into *Escherichia coli* DH5α cells. Colonies containing vector were selected on LB-plus Kanamycin (100 μg/ml) plates. Plasmid DNA was digested with restriction endonucleases to determine the insertion size and was further verified by commercial sequencing. Histidine-tagged antigens were expressed and purified as described previously [27].

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**Figure 1** Structure of the reconstructed region of pCambia 2300-Patatin plasmid (A) and treatment schedules (B).
Plant transformation and growth of tubers

Recombinant plasmids (antigen-Cambia 2300-Patation I plasmid) were mobilized into Agrobacterium tumefaciens LBA4404 (gift from Prof. Zhong-Ping Lin) using the freeze–thaw method. The structures of plasmids in the transformed LBA4404 lines were verified by restriction digestion (data not shown). Desiree potatoes (Solanum tuberosum) were transformed by modified leaf-disc co-cultivation methods using the Agrobacterium strains described above. The leaves with tiny cut on the edge were immersed for 30 min in a suspension of A. tumefaciens grown to early log phase and then co-cultivated on Petri dishes with MS agar medium containing 6-benzylaminopurine (0.1 mg/l) and 2 mg of 1-naphthaleneacetic acid (5 mg/l) in darkness. After 48 h, the leaves were transferred to MS medium containing glucose (1.6%), trans-zeatin (1.5 mg/l), gibberellin (0.02 mg/l), and cephalosporin (100 g/l, Sigma, St Louis, USA) in a growth chamber with 16 h light of a day at 25°C for about 8 weeks until the new plantlets regenerated from the callus. The regenerated shoots were transferred to rooting medium (MS agar medium supplemented with 50–100 mg/l kanamycin and 500 mg/l cephalosporin). Plantlets rooting on selection medium with kanamycin (Sigma) in increasing concentration gradient from 50 to 100 μg/ml were clonally propagated and tested for the presence of inserted antigen-DNA in potato genome by PCR analyzation, total genomic DNA from wild-type potatoes as a negative control (Fig. 2). The positive plants were then transplanted to soil and grown in a greenhouse for 2–3 months. The mature tubers were stored at 4°C until use.

Total protein extraction and western blot analysis

For analysis of the transgenic plants, soluble protein extracts from potato tubers were obtained by the BPP protocol [28]. The protein concentration was determined by Bradford method. Total soluble proteins (3 μg) were used for western blot analysis. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto Immobilon-P membrane (Millipore, Billerica, USA) by electro-blotting. The detection of the transgenic protein was performed with the polyclonal antibodies (1.5 mg/ml, 1000/C2) derived from mice immunized with the combined plasmids (100 μg of each plasmid encoding Ag85b, MPT83, MPT64, and ESAT6, dissolved in saline). The secondary antibody was a horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Ig) G (0.8 mg/ml, 5000/C2; Jackson, West Grove, USA). Detection was carried out using the ECL detection system (CWBOIO, Beijing, China).

Animals

Specific pathogen-free C57BL/6 female mice were obtained from the Animal Center at the Academy of Military Medical Sciences (Beijing, China). The mice, maintained in barrier conditions at the Laboratory Animal Centre, Peking University, (Beijing, China), were 6–8 weeks old at the time of vaccination and were fed with commercial mouse feed and water. Mice were monitored on a daily basis for signs of weight loss and physical condition.

Preparation of potato vaccine

The potatoes were peeled and cut into cubes. Transgenic and wild-type potato cubes were pooled into separate bowls. The cubes were mixed, immediately ground using a blender, and the filtrate was collected before being administered via oral gavage.

Immunization

For oral-gastric dosing, test animals were transferred to individual cages and allowed to fast for 8 h before introduction of the test diet. The mice had continuous supply of water. The groups were summarized in Table 1. Immunizations were given on Days 0, 7, 14, and 28 (Fig. 1(B)), and the fourth vaccination was for an immune boost. Mycobacterium bovis BCG strain was obtained from...
the Center of Tuberculosis Research (Beijing, China) and used as a positive control. Consistent with the experimental group, the BCG group were fed orally with 1.8 × 10^7 CFU BCG in 100 µl saline per mouse. The mice of combined-plant vaccine group were fed with 1 ml of the concentrated transgenic potato extract (~10 g of raw potatoes in total) per mouse per feeding. The negative control mice received wild-type potato extract. Another nine mice were fed with 0.9% (w/v) NaCl solution as a control. Immunization was performed using a 1-ml syringe fitted with a gavage needle. Random selections of three or five mice from each group were sampled for immunogenicity.

Specific antibody analysis using indirect enzyme-linked immunosorbent assay
Immunized mice were bled from the retro-orbital vein using capillary pipettes at 2 weeks after each vaccine administration. Sera were collected from five mice immunized with the transgenic potato extract containing the mixed antigens. Antigen-specific antibody titers of serum from each group were analyzed by indirect enzyme-linked immunosorbent assay (ELISA). Briefly, microtiter plates (NUNC, Roskilde, Denmark) were coated overnight at 4°C with 100 µl of the 5 µg/ml purified antigens (Ag85B, MPT83, MPT64, and ESAT6) or 10 µg/ml tuberculin PPD (purified protein derivative, for BCG group) in carbonate/bicarbonate buffer (pH 9.6) [24,29,30]. In addition, sera from five mice were analyzed in triplicate wells. The endpoint titer was defined as the highest dilution of serum that gave an OD_{450} exceeding 0.050, and was 2 folds higher than that of the matched dilution of unvaccinated mouse sera.

Measurement of cytokines
Spleens from three vaccinated mice were removed aseptically 2 weeks after the last administration via oral gavage. The spleen cells collected from three mice were adjusted to a concentration of 4 × 10^6 cells/ml before being cultured in round-bottomed microwell plates (NUNC) in RPMI 1640 medium supplemented with L-glutamine, 50 µM 2-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% (w/v) heat-inactivated fetal calf serum (Gibco-BRL, Rockville, USA). From each group, 180 µl of cell suspension was added to 20 µl of the purified recombinant antigen (final protein concentration, 5 µg/ml) or tuberculin PPD (purified protein derivative) (final protein concentration, 10 µg/ml). Cells were incubated at 37°C in a humidified CO₂ incubator, and supernatants were harvested after 72 h. Supernatants from two separate wells were pooled and frozen at –80°C. The concentrations of the cytokines IFN-γ, IL-2, and IL-4 were measured using Cytokine ELISA MAX Set Deluxe kits (BioLegend, San Diego, USA).

Flow cytometric analysis
Two weeks after the last vaccination, peripheral blood mononuclear cells (PBMCs) from heparinized orbital blood and splenocytes in each group were isolated by Ficoll-Hypaque density-gradient centrifugation. CD4^+ and CD8^+ T cells were selected from splenocytes using mouse CD4 (L3T4) or CD8 (Lyt-2) antibody-conjugated Dynabeads slurry (Dynal Biotech ASA, Norway) according to the manufacturer’s instructions. Both PBMCs and the selected T cells were incubated for 48 h at 37°C in 5% (w/v) CO₂ with mixed antigens (5 µg/ml for each antigen) or without (control), respectively. Cells were then washed in cold phosphate-buffered saline buffer (PBS). The PBMCs were incubated with rat anti-mouse CD4-PE/Cy5 mAb, rat anti-mouse CD8-APC mAb (Serotec, Oxford, UK), while the selected T cells were treated with monoclonal antibodies against CD44 (Serotec) in PBS buffer with 10% (w/v) fetal bovine serum for 30 min at 4°C in the dark. Finally, cells were washed, resuspended in PBS buffer, and analyzed using BD FACS Calibur flow cytometer (BD Corporation, San Jose, USA).

Statistical analysis
The statistical significance of data was analyzed by one-way analysis of variance followed by Tukey’s test using the SigmaStat 3.5 program (Systat Software, Inc., Richmond, USA).

Results
Nucleic acid analysis and detection of transgenic protein expression in potato tubers
Bands of 996-bp, 681-bp, 687-bp, and 333-bp, corresponding to the expected size of the gene, were amplified by PCR from DNA samples from kanamycin-resistant plants transformed with the antigen–Cambia 2300–Patation I plasmid (Fig. 2). No band was amplified from DNA samples from non-transformed plants. This demonstrated that the putative transgenic plants germinated on kanamycin indeed possess the desired gene. Meanwhile, transgenic protein expression in potato tubers was detected by western blot. As the results show, the specific band of each protein was observed in the soluble total protein samples from the transgenic potato tubers using the polyclonal
antibody (Fig. 2). No band was detected from the soluble total protein samples from non-transgenic potato tubers.

**Combined plant-made vaccines induce higher antigen-specific Th1 cytokine production**

As in cases of TB infection, an important marker of Th1-mediated acquired immunity is the production of Th1 cytokines. IFN-γ is known to be essential in the control of TB in experimental mice, and IL-2, produced by antigen-activated CD4+ T cells, has been linked to enhanced memory/effector function. We therefore evaluated the production profile of these cytokines by capture ELISA. Two weeks after the last administration via oral gavage, splenocytes of treated mice were stimulated with the four antigens, Ag85B, MPT83, MPT64, and ESAT6. Then the levels of IFN-γ and IL-2 production in cell culture supernatant were determined by ELISA. Mice treated with the combined plant-made vaccines showed a significantly higher level of IFN-γ and IL-2 production in response to all four proteins (P < 0.01). BCG-vaccinated mice produced a significantly higher level of IFN-γ and IL-2 in response to PPD compared with mice treated with saline solution (P < 0.01). In addition, low levels of cytokines were detected in the wild-type and saline groups [Fig. 3(A,B)]. Meanwhile, the induction of Th2 cytokine, IL-4, from various group were very low, although the expression patterns were similar to that of IFN-γ and IL-2 [Fig. 3(C)]. Our results indicated that the combined plant-made vaccines induced stronger Th1-biased responses.

**Combined plant-made vaccines induce a higher mean percentage of CD4+ and CD8+ T cells**

The most effective vaccination strategies in animal models are those that stimulate both CD4+ and CD8+ T cells to produce Th1-associated cytokines. Assays were carried out using T cells in the presence of PBMCs and the mixed four antigens to determine T-cell-subset-restricted responses. The mean percentages of CD4+ and CD8+ T cells in mice treated with combined plant-made vaccines were significantly higher in PBMC cultures re-stimulated with the mixture of four specific antigens (CD4+, 36.1 ± 1.9; CD8+, 23.4 ± 1.8) compared with the saline group (CD4+, 28.7 ± 2.6; CD8+, 14.2 ± 1.3) 2 weeks after the final vaccination (P < 0.01). Moreover, the mean percentage of CD4+ T cells in combined plant-made vaccines was up to 1.2 fold higher than the saline group (Fig. 4). The CD4+ and CD8+ T cells in the BCG group (CD4+, 33.2 ± 2.2; CD8+, 21.3 ± 1.7) had an intermediate mean percentage. In addition, lower mean percentages of CD4+ and CD8+ T cells were detected in the wild-type group. These findings indicated that the majority of animals generated a predominantly CD4+ T-based T-cell-specific response.

**Combined plant-made vaccines induce higher activated T-cell expression of CD44**

The memory/activated T cells (expression of CD44), which mediate the long-lived host response against TB, were characterized in mice with a different treatment. Mice treated with the combined plant-made vaccines had T-cell populations with a significantly higher expression of CD44.
(P < 0.001) at 2 weeks after injection. In contrast, the majority of T cells presented no significantly higher expression of CD44 in mice treated with the wild-type group than those from mice treated with the saline group (Fig. 5).

**Combined plant-made vaccines induce stronger humoral immune response with antibodies as characterized by both IgG and IgA type after vaccination**

Antibody titers against the four mycobacterial antigens used in this study were determined in sera harvested from five immunized mice 2 weeks after each injection. The total amount of IgG provided a good indicator of the immune response generated in mice. As seen in Table 2, all mice treated with the combined plant-made vaccines induced higher total IgG and IgA antibody levels than those induced by the wild-type plant vaccine. Substantial differences in antibody titers were noted between the two groups. Specific IgG antibodies responding to Ag85B and MPT64 could be detected between 1:800 and 1:3200 dilution in mice treated with combined plant-made vaccine 2 weeks after the third and fourth immunization. The MPT64-specific IgA antibody levels reached 1:3200 at 2 weeks after the third immunization. The saline group elicited no detectable antibodies throughout the entire experiment (Table 2). In general, the antibody concentrations induced in response to combined plant-made vaccines reflected the level of antigenic expression seen in vitro.

**Discussion**

The BCG vaccine is effective in protecting against the infant forms of TB, but has a limited and variable effect in adults against the pulmonary form of the disease, making the development of an improved vaccine critical in reducing the 8–10 million new TB infections per year [31,32]. An improved vaccine against TB should be economical and easy to administer so as to encourage and facilitate its use in low-income countries where the vaccine is most needed. In this study, we developed a new combined plant-made vaccine delivery system and demonstrated a strong cellular response against the expressed candidate antigens. The immunity against mycobacterial infection is attributed to cellular immunity. The subsets of Th cells are related to the types of immune response. Th1 response, which is dominated by antigen-specific T lymphocytes that produce IFN-γ and are cytotoxic towards infected cells, is probably required for maximally effective anti-mycobacterial immunity in mice and humans [33–36]. Obviously, it is most important to ensure a Th1 response that induces IFN-γ and other cytokines [33]. Our studies indicated that combined plant-made vaccines stimulate a higher concentration of IFN-γ and IL-2. The ESAT6 and MPT83 antigen-specific IFN-γ resulted in 600–700 fold higher response than those using the wild-type plant vaccine (Fig. 3). Although IFN-γ production by CD4⁺ T cells is essential, the CD8⁺ cell response is also important. β₂-Microglobulin-deficient mice, which lack functional CD8⁺ T cells, are highly susceptible to M. tuberculosis [33–36]. Our data demonstrated that a higher mean percentage of CD4⁺ and CD8⁺ T cells were obtained (Fig. 4). The humoral response is indispensable [37]. First, opsonization of mycobacteria might improve phagocytosis by neutrophils that are more effective in killing intracellular M. tuberculosis. Second, macrophage

![Figure 4](https://academic.oup.com/abbs/article-abstract/44/10/823/959)

*Figure 4* The percentage of CD4⁺, CD8⁺ T cells in mouse PBMCs at 2 weeks after the last immunization T-cell subpopulations in mouse PBMCs were determined by a FACSCalibur flow cytometer using anti-mouse CD4⁻-PE/Cy5 and CD8⁻-APC-MAb. The mean percentage of CD4⁺ or CD8⁺ T cells was significantly greater than in the saline group (**P < 0.01**). Each column represents the mean ± SD from triplicate measurements.

![Figure 5](https://academic.oup.com/abbs/article-abstract/44/10/823/959)

*Figure 5* Flow cytometry analysis of memory T cells production of immunized mice Single-cell suspensions of splenocytes (1 × 10⁷ cells/well) were prepared from three mice at 2 weeks after the last vaccination. CD4⁺ or CD8⁺ T cells were selected, then treated with MAb against CD44 and analyzed on a FACSCalibur flow cytometer to quantify the CD44⁺⁺ cell populations. The data reflect the mean ± SD from triplicate measurements; experiments were performed twice. ***P < 0.001, compared with the saline group.
uptake of mycobacteria through Ig receptors could increase intracellular killing by the usual host for mycobacterial persistence. Third, Ig-induced redirection of uptake and/or cellular activation in antigen-presenting cells could increase the induction of mycobacteria-specific T-cell responses. Our data showed that higher total IgG and IgA antibody levels were induced by the combined plant-made vaccines than by the wild-type plant vaccine group (Table 2).

Specifically, mice treated with combined plant-made vaccine generated antigen-specific and high IgG titers when measured in 2 weeks after the third immunization, and exhibited even higher IgG response against the four antigens except for ESAT6 antigens 2 weeks after the fourth immunization (Table 2). Meanwhile, high IgA titer against the four antigens except for Ag85B antigens was induced in the transgenic plant-immunized mice when measured in 2 weeks after the second and third immunization (Table 2). Moreover, the combined plant-made vaccines led to enhanced antigen-specific CD4CD44high cells (Fig. 5). This effect may also be attributed to the fact that more Th cells were activated by APC at the time of immunization because the initial immune response intensity usually has an important effect on memory cell production. Our results indicated that robust CD8+ and CD8CD44high cell responses were obtained (Figs. 4 and 5). In addition, the non-specific T-cell populations from each immunized group stimulated without mixture antigens was similar to the saline-immunized and mixture antigen-treated group (data not shown). Collectively, the above observations indicated that our combined plant-made vaccines induce a robust (qualitative and quantitative) antigen-specific IFN-γ-produced Th1 immune-response. The long-term goal of our studies is to find a stable, effective, and long-lasting oral vaccine.

### Table 2 Serum IgG/IgA titer analysis of immunized mice 2 weeks after each vaccination

<table>
<thead>
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<th>Antigen</th>
<th>IgG titers</th>
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<th>IgA titers</th>
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<tr>
<td></td>
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<td>Second</td>
<td>Third</td>
<td>Fourth</td>
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<td>Transgenic plants</td>
<td>Ag85B</td>
<td>1 : 200</td>
<td>1 : 800</td>
<td>1 : 1600</td>
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<td></td>
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<td>Wild-type plants</td>
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<td>All antigens</td>
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*Mixture sera samples (five mice per group) were analyzed for Ag85B, MPT83, MPT64, and ESAT6-specific IgG and IgA antibodies by ELISA. Antigen-specific total IgG and IgA endpoint titers were determined at 2 weeks after vaccination. The four antigens induced the same antibody titers in two independent experiments.

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