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Immunogenicity of recombinant bacterial antigens expressed as fusion proteins in transgenic rice seeds

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Abstract

Rice-based vaccines do not require high-cost purification. They are stable at room temperature, can eliminate the risk of attenuated vaccine strains, and are resistant to gastrointestinal degradation. We tested the applicability of an oral delivery system for tuberculosis (TB) and cholera antigens in transgenic rice for induction of immune responses in the mucosal compartment as well as in the systemic circulation. For vaccine development, we selected mycobacterial Ag85B antigen and immunoprotective P4 epitope of TcpA fused to the nontoxic cholera toxin B (CTB) subunit for immunization against TB and cholera, respectively, in independent constructs. The expression levels of CTB, CTB-TcpA, and CTB-Ag85B in transgenic lines containing stably integrated, chimeric genes showed up to 0.64%, 0.34%, and 0.02% of total rice seed protein, respectively. Oral immunization of mice with each of the three seed lines resulted in significantly increased levels of both anti-CTB IgG and IgA responses in the serum and IgA responses in the bronchoalveolar lavage (BAL) fluid. This indicated the capacity for oral immunization to elicit immune responses in the respiratory mucosal compartment. Plant-expressed TcpA could be detected in immunoblot analysis by using TcpA-specific commercial antibody, while there was no recognition of rice-expressed Ag85B by the commercial antibody raised against the latter antigen, where both antibodies were produced against the antigens expressed in the bacterial system. This study focused on identifying antigens resistant to both posttranslational modifications in plants and immunogenic under the proposed delivery system in animals for boosting the mucosal and systemic humoral immune response against enteric as well as respiratory pathogens.

Key words: Ag85B, cholera, oral vaccines, TcpA, transgenic rice, tuberculosis

Introduction

Vaccines have revolutionized the prevention of many infectious diseases. Orally administered edible vaccines produced or obtained using transgenic plant technology are one of the latest additions to the vaccine research to overcome the limitations of conventional vaccines (Tiwari et al., 2009). Tuberculosis (TB) is the second most deadly infectious disease in human after AIDS, which is caused by a single infectious agent, *Mycobacterium tuberculosis* (Tang et al., 2016). An estimated 6 million new TB cases, with 1.5 million associated deaths, were reported in 2014 alone (WHO, 2015). In endemic regions, cholera causes diarrhea and severe dehydration in humans in a distinctive seasonal pattern. It is predominantly triggered by *Vibrio cholerae* O1, with an estimated 1.4 billion people at risk of infection (Ali et al., 2012). Both microorganisms primarily target high-risk, vulnerable populations and constitute substantial roadblocks to healthy development in many socioeconomically underprivileged societies. Intrinsically, the production of highly immunogenic and easily deliverable vaccines is crucial to circumvent the loss of productivity of patients and prevent morbidity (Holmgren and Svennerholm, 2012).

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Contrary to other routes of vaccine delivery, oral vaccination utilizes the gastrointestinal tract where orally delivered antigens are processed and absorbed through the mucosal-epithelial barrier. As mucosal immunity provides the first line of defense against the microbial invaders of gastrointestinal, respiratory, and genitourinary systems through the production of secretory IgA, it would be effective to develop vaccines to strengthen the mucosal defense mechanism (Nochi et al., 2007). Both *M. tuberculosis* and *V. cholerae* affect different parts of the mucosal surfaces, with the former targeting the lungs and other systemic organs, while the latter infects the intestinal mucosa (Lycke, 2012). In cholera, oral vaccination has long been considered more satisfactory for protection than the systemic route (Clemens and Holmgren, 2014; Svennerholm et al., 1983), whereas studies on mouse models using *M. tuberculosis* have shown that the mucosal (intranasal) route of vaccination is more effective than the systemic one (Nuermberger et al., 2004; Wang et al., 2004), highlighting the importance of a route of administration.

The lower costs of a plant-based antigen production make it suitable for large-scale accessibility as it is stable at room temperature for at least 18 months, as in the case of rice-derived CTB subunit (Nochi et al., 2007), and can therefore be transported at low cost to remote areas of afflicted populations. Moreover, plant-expressed antigens do not pose the risk of causing disease, which may be a concern with traditional vaccines that utilize killed or attenuated organisms for immunizations. A lower cost of production of antigens in plants is also likely to render the vaccines available at low prices in regions which require affordable solutions to decrease the disease burden (Streatfield et al., 2001; Walmsley and Arntzen, 2000). Thus, these edible vaccines offer a practical and cost-effective means of vaccinating large populations against various infections, and a successful trial of rice-based oral delivery of fusion antigens may provide insights into the development of new vaccines not only against TB and cholera, but also other mucosal or systemic diseases.

For vaccination against TB, we selected a highly immunogenic major secretory protein of *M. tuberculosis*, antigen 85 subunit B (Ag85B) (Kaufmann et al., 2010; Lozes et al., 1997), whereas for cholera the P4 epitope of the major subunit of the *V. cholerae* O1 toxin coregulated pilus antigen (TcpA) was chosen (Herrington et al., 1988; Kiaie et al., 2014). Another critical component of oral vaccination is the selection of an efficient mucosal adjuvant, as its absence frequently results in the induction of tolerance (Lu et al., 2002) The *V. cholerae* -derived B subunit of cholera enterotoxin (CTB) is a strong immunological adjuvant capable of inducing specific serum and mucosal immune responses in mice (Holmgren et al., 1993; Qiu et al., 2014) and has previously been expressed in rice (Nochi et al., 2007), with accumulation levels reaching ~2.1% of total seed protein levels. In this sense, CTB can be used in combination with other proteins that are integrated in the same recombinant molecule to act as both adjuvant and carrier for these antigens.

Although chimeric structures comprising CTB-P4 epitope have been expressed in tomato (Sharma et al., 2008), their immunogenicity was not tested in animals and in spite of promising reports of plant-made oral vaccines for *M. tuberculosis* in murine models (Rigano et al., 2006; Zhang et al., 2012), no attempts have been made to incorporate mycobacterial antigens in rice. We aimed to test the efficiency of orally delivered TB and cholera antigens expressed and accumulated in rice seeds to strengthen mucosal immune protection and to promote the concept of inducing immunity in the gastrointestinal and respiratory tract.

Materials and methods

Cloning of rice glutelin GluB-1 promoter sequence

We constructed a binary vector, suitable for *Agrobacterium*-mediated plant transformation, containing the rice endosperm-specific promoter, *GluB-1* (LOC_Os02g 15169), to control downstream gene expression as described by Nochi et al., (2007). The 2.4-kb *GluB-1* promoter sequence with a signal peptide was amplified from rice genomic DNA (originating from japonica cultivar *Oryza sativa* L. cv. Nipponbare) using primers inclusive of recognition sites for the restriction enzymes *EcoRI* (5′-GCGTGAATTC**CAGATTCTTGCTACCAAC**-3′) and *PstI* (5′-TATACTGCAG**GGCCATAGAACCATGGCA**-3′). The conditions for amplification were as follows: 95°C for 1 min, 60.2°C for 1 min and 72°C for 2.30 min for a total of 35 cycles. Directional cloning was performed by ligating the promoter insert in between the corresponding *Eco*RI and *Pst*I recognition sequences contained within the multiple cloning site (MCS) of the binary

vector pCAMBIA 1305.1 (Cambia, Australia). The resultant construct was designated pCAMBIA_GluB1 and the recombinant vaccine sequences were subsequently cloned directly downstream of the *GluB-1* promoter in this vector.

Cloning of recombinant vaccine sequences

The plant-codon optimized recombinant gene sequences for the adjuvant CTB (GenBank accession no. K01170), CTB fused at the C-terminal to the full Ag85B subunit (UniProt accession no. P0C5B9), and CTB fused to the P4 epitope of TcpA (GenBank accession no. U09807; 145-168 amino acids) were commercially synthesized in three independent vectors (Integrated DNA Technologies, USA). All three genes featured the SEKDEL signal 3′ to the antigen, followed by the *GluB-1* termination sequence. Additionally, for both CTB-Ag85B and CTB-TcpA-P4, the adjuvant and antigen were separated by a 7-amino acid glycine hinge. The restriction enzymes for cloning were selected to permit ligation directly downstream of the *GluB-1* promoter. The sequences (1000 bp for CTB; 1076 bp for CTB+TcpA-P4; and 1958 bp for CTB+Ag85B) were amplified separately from the three vectors using primers inclusive of recognition sites for *Pst*I (5′-GCTATACTGCAGACCCCGC AGAACATCAC-3′) and *Hind*III (5′-CGCCGTAAGCTT CTCTTAACTTTACCTATG-3′) using the following conditions: 95°C for 1 min, 60.2°C for 1 min and 72°C for 2 min for a total of 35 cycles. The inserts were directionally cloned separately into pCAMBIA_GluB1, and positive clones were identified via PCR amplification, restriction digestion, and plasmid DNA sequencing (1st BASE, Malaysia). The constructs were then individually electroporated into *Agrobacterium tumefaciens* strain LBA4404, with plasmids from the transformed colonies subjected to screening by PCR and restriction digestion (Sambrook, 1987).

Generation of transgenic rice plants

The binary vectors containing recombinant gene sequences within the transfer DNA (T-DNA) were introduced into the genome of a wild-type (WT) rice variety *Oryza sativa* (indica cv. Binnatoa) by the *Agrobacterium tumefaciens* LBA4404-mediated transformation method (Islam et al., 2009; Rasul, 1997). All the vectors had the reporter gene, β-glucuronidase (GUS), with the catalase intron driven by the constitutive CaMV35S promoter within the T-DNA that integrates into the plant genome (Fig. 1). Therefore, a positive GUS assay from an isolated plant tissue indicates integration of the T-DNA into the plant genome, since only the plant cells have the capacity to splice out the intron and produce the GUS enzyme. Shoots regenerated from the transformed calli, designated as T_0 plants, were transplanted to soil and grown to maturity in a confined net house (the average temperature and humidity of the net house were 28°C and 87%, respectively). Seeds were harvested (105-110 days) from positive T_0 plants, designated as T_1 and verified for gene expression by histochemical GUS (β-glucuronidase reporter system) assay (Jefferson, 1987). GUS-positive seeds were germinated and propagated up to T_2 and T_3 generations. T_2 and T_3 seeds from T_1 and T_2 plants, respectively, were evaluated for the production of recombinant protein in the endosperm.

PCR analysis of transgenic plants

Genomic DNA was isolated from transgenic rice leaves (T_0-T_2) using a silica-based extraction method as described previously (Li et al., 2010). The presence of the CTB, CTB-Ag85B, and CTB-TcpA-P4 recombinant genes was determined by PCR analysis using a forward flanking primer within the *GluB-1* promoter (5′-TTGC AAAGTTGCCTTTCCTT-3′) and a reverse *GluB-1* terminator-specific primer (5′-CTCTTAACTTTACCTATG-3′). For PCR, 50 ng of genomic DNA was used as a template and the conditions were as follows: 95°C for 1 min, 56°C for 1 min and 72°C for 2 min for a total of 35 cycles. Total RNA was isolated from mature T_2 seeds using an established protocol (Sangha et al., 2010), but using extraction buffer components from the method by Suzuki and coworkers (Suzuki et al., 2008). First-strand cDNA was synthesized from 5 μg of total RNA using the kit protocol for SuperScript® III First-Strand Synthesis System for RT-PCR (Life Technologies, USA). The expression of the mRNA transcripts was verified using a forward adjuvant-specific primer (5′-ACCCCGCAGAA CATCAC-3′) and a reverse primer located within the 3 UTR region of the *GluB-1* terminator, but before the putative transcription termination site (5′-TCGAACAG AATCACCACCAA-3′). For RT-PCR, reverse-transcribed cDNA was used as template and subjected to thermocycling conditions as follows: 95°C for 1 min, 58.9°C for 1 min and 72°C for 1.30 min for a total of 35 cycles.

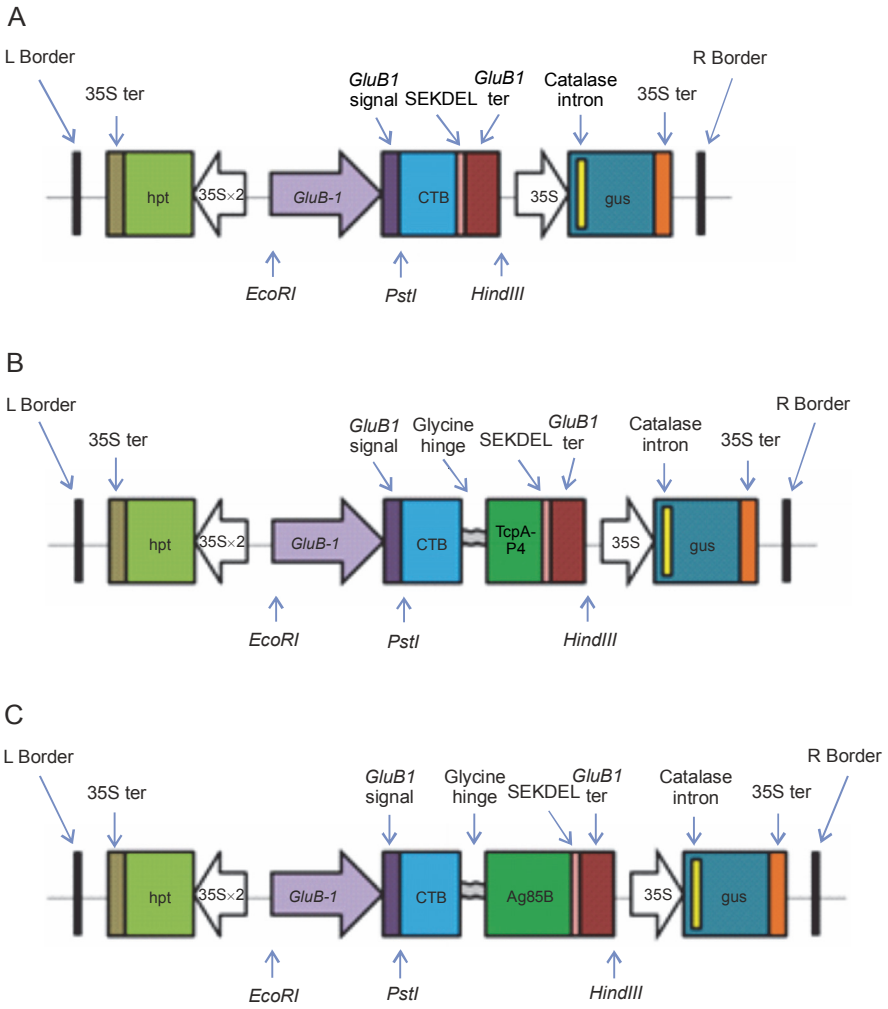

Fig. 1. Diagrammatic representation of T-DNA regions of plant expression vectors: A) pCAMBIA_GluB1_CTB, B) pCAMBIA_GluB1_CTB_TcpA-P4 and C) pCAMBIA_GluB1_CTB_Ag85B. hpt, hygromycin phosphotransferase gene; gus – beta-glucuronidase gene from *E. coli*; 35S – CaMV35S promoter; 35Sx2 – CaMV35S promoter with a double enhancer; SEKDEL – endoplasmic retention signal

Protein extraction and detection of recombinant proteins

Total seed proteins (TSP) were extracted from the T_1, T_2, and T_3 seeds of transgenic rice plants (Nochi et al., 2007) and used for the immune-detection of recombinant proteins. About 0.1 g of rice seeds were ground to a fine powder-like consistency using mortar-pestle, strained using a fine mesh, and then the powder was mixed with protein extraction buffer (4% sodium dodecyl sulfate (SDS), 8 M urea, 50 mMTris-HCl (pH 6.8), 5% 2-mercaptoethanol, 20% glycerol). The mixture was vortexed for 30 min at room temperature and the supernatant was centrifuged (12,000 rpm for 45 min). About 5 µg of TSP extracts, determined using standard Lowry protein assays, were separated on 13.5% SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions after boiling the samples with 10 ng of purified recombinant CTB (A. M. Svennerholm, University of Gothenburg, Sweden), 10 ng of Ag85B antigen (BEI Resources, USA), or 10 ng of TcpA protein (Asaduzzaman et al., 2004). Gels were transferred to a nitrocellulose membrane, air-dried, blocked with 5% skimmed milk in phosphate buffer saline (PBS) for 60 min, and incubated 90 min with rabbit polyclonal anti-CTB (1:50 000; CalBioreagents, USA), rabbit polyclonal anti-Ag85B (1:50 000; Abcam, UK) or rabbit monoclonal anti-TcpA (1:30 000; gift from E. Ryan, Massachusetts General Hospital, USA) antibodies. The membranes were subsequently washed 3 times in PBS with 0.05% Tween 20 for 10 min and incubated 90 min with goat anti-rabbit IgG-horseradish peroxidase (HRP; Abcam, UK) and washed again thrice in the same

way. The membranes were then incubated with enhanced chemiluminescence (ECL) western blot detections reagents (GE Healthcare, UK) for 2 min and exposed to an X-OMAT radiographic film. Images of the protein bands were taken using a scanner (HP Scanjet G2410) and analyzed for comparative densitometry data using the image-processing program ImageJ (National Institutes of Health, USA).

Oral immunization

The studies were performed using 6- to 8-week-old female BALB/c mice raised at the Animal Resource Branch of the icddr,b. Experimental and management procedures were approved by both the Research Review Committee and the Animal Experimentation Ethics Committee of the ICDDR,B.

Three cohorts of BALB/c mice (age 6-8 weeks, n = 6-8) were immunized through the oro-gastric route with seed powder (ground using mortar-pestle and then strained using fine strainer) from nontransgenic wild-type Binnatoa (120 mg/mouse), transgenic CTB (120 mg/mouse), transgenic CTB-Ag85B (120 mg/mouse), or transgenic CTB-TcpA-P4 (120 mg/mouse) solubilized in 500 µl PBS. A maximum four mice from the same vaccine group were placed in one cage, and each mouse was fed with 120 mg seed powder as 100 mg/mouse dose was shown to elicit serum IgG and fecal IgA responses (Nochi et al., 2007). For the non-rice control groups, mice were orogastrically immunized with either purified rCTB antigen (25 µg/mouse) or PBS alone. Immunizations were performed on days 0, 10, 20, 30, and 40 and serum was collected on days 0, 10, 20, 30, 40, and 47. BAL was retrieved on day 47 from the trachea of euthanized mice using a syringe-aided resuspension of PBS buffer.

Antibody responses

For CTB-specific ELISAs, plates (Nunc, Denmark) were coated with 0.3 nmol of ganglioside GM1/ml, sequentially followed by recombinant CTB (0.5 µg/ml) and 100 µl sera from immunized mice (diluted 1 : 100 in 0.1% bovine serum albumin (BSA) in PBS-0.05% Tween). For TcpA- and Ag85B-specific ELISAs, plates were coated with either 1 µg/ml purified TcpA in PBS or 1 µg/ml Ag85B antigen, respectively, and blocked with 1% BSA-PBS prior to incubation with mice sera as described above. The plates were incubated for 90 min at 37°C, following which goat anti-mouse IgG, IgM, and IgA (Southern Biotech, USA) antibodies conjugated to HRP were applied separately. After incubation period, the plates were developed with 1 mg/ml ortho-phenyle diamine (Sigma-Aldrich, USA) in 0.1 M sodium citrate buffer (pH 4.5) and 0.04% hydrogen peroxide, after which readings were obtained kinetically at 450 nm for 5 min. The maximal rate of change of optical density per minute was calculated in milli-absorbance units. ELISA protocol was followed using BAL fluid (diluted 1 : 2 in 0.1% BSA in PBS-0.05% Tween) to test mucosal IgA responses.

For dot blots, nitrocellulose membranes were soaked in PBS and allowed to dry for 5-30 minutes, following which 1 µl each of the positive control (Affinipure goat anti-human IgG 1.8 mg/ml; Jackson ImmunoResearch, USA), target antigen (TcpA antigen 1 mg/ml), and the negative control lacking cross-reactivity (*V. cholerae* O1 Ogawa Lipopolysaccharide 1 mg/ml) were applied. The membranes were blocked with 1% BSA-PBS for 30 min at room temperature with gentle shaking. After washing with PBS, samples from the immunized mice were diluted (1:50 for serum and neat for BAL) in 0.1% BSA-PBS with 0.05% Tween and incubated for 3 hours at room temperature, prior to a round of washing with PBS-Tween (0.05%). Polyclonal rabbit anti-mouse immunoglobulin HRP (Dako, USA) was added afterward and the membranes were incubated further for 1.5 hours at room temperature before undergoing development using 4-chloro-1-naphthol and H_2O_2 solution (Sigma-Aldrich, USA) for approximately 10 min.

Pepsin digestion

Transgenic seed powder (10 mg) and purified rCTB (15 µg) were incubated with 0.5 mg/ml pepsin (Sigma-Aldrich, USA) in 0.1 ml of 0.1 M sodium acetate buffer (pH 1.7) for 60 min at 37°C with gentle shaking. Digestion reactions were terminated with the addition of protein extraction buffer as described above and the mixtures were subjected to Western blot and densitometric analyses as described earlier to evaluate protein degradation. ImageJ software was used for densitometric analyses.

Protein identification

The CTB-Ag85B insert was amplified from both leaf genomic DNA and reverse-transcribed seed cDNA, following which the PCR products were subjected to se-

quencing (1st Base, Malaysia). Once the integrity of the transgene was established at both DNA and RNA levels, the CTB-Ag85B fraction of TSP was submitted to two different companies for protein identification by mass spectrometry. Protein extracts were separated on a 13.5% SDS-PAGE gel and stained with Coomassie blue, and gel slices corresponding to the expected ~50 kDa band region were excised. Protein samples were digested with trypsin at Proteomics International (Australia), whereas proteolysis at Proteome Factory (Germany) involved the use of either trypsin or thermolysin. The resulting peptides were analyzed by electrospray ionization mass spectrometry using a nano HPLC system coupled to a 5600 TripleTOF mass spectrometer (AB Sciex; Proteomics International) or an Orbitrap XL mass spectrometer (ThermoFisher; Proteome Factory). Peptides were trapped and separated using water/acetonitrile/0.1 formic acid using either a linear gradient (Proteomics International, Australia) or a gradient from 5% to 35% acetonitrile (Proteome Factory, Germany). The spectra were analyzed to identify protein sequences of interest using the Mascot search engine's MS/MS ion search (Matrix Science, UK) using Ludwig NR database with taxonomy limited to eubacteria (Proteomics International, Australia) or a protein database to which the fusion protein sequence was appended (Proteome Factory, Germany).

Data analysis

Comparisons were made based on the magnitude of the elicited immunological responses. The nonparametric Mann-Whitney U-test was performed to compare immunological responses of mice immunized with different vaccine groups (CTB, CTB-TcpA, or CTB-Ag85B) with mice fed with non-transgenic WT control on different days. All reported P values are two-tailed, with a cut-off value of ≤ 0.05 considered to be a threshold for statistical significance. GraphPad Prism 5.0 was used for various analytical purposes.

Results and discussion

Development of transgenic rice plants expressing recombinant proteins in seeds

Constructs for the expression of fusion proteins were engineered (Fig. 1) by first cloning the 2,335 bp *GluB-1* promoter together with its 72 bp signal peptide into a binary vector. To achieve a high expression and accumulation of the inserted antigen in rice seed, a highly active endosperm-specific glutelin *GluB-1* promoter was chosen to drive expression of the synthetic vaccine sequences (Katsube et al., 1999; Qu et al., 2002). All sequences that were not native to plants were codon-optimized for optimal expression in rice seeds, as it has been reported that such an approach results in increased protein expression (e.g., optimizing the codon usage of CTB for expression in rice achieved accumulation levels of ~2.1% of TSP) (Daniell et al., 2001; Nochi et al., 2007).

Each recombinant gene was cloned directly downstream to this promoter and consisted of the following common elements: the 312 bp CTB adjuvant affixed to a 21 bp glycine hinge, followed by 21 bp endoplasmic reticulum retention signal SEKDEL and stop codon fused to 629 bp *GluB-1* terminator region. The presence of the SEKDEL signal, along with the *GluB-1* signal peptide containing the entire 5′ untranslated region and the 3′ untranslated region responsible for directing mRNA to cisternal ER, may together be involved in the targeting of peptides to seed protein bodies (Schouten et al., 1996; Wandelt et al., 1992). For the two chimeric genes, either the 72 bp P4 epitope of TcpA or 975 bp Ag85B sequence was separately synthesized at the 3′ terminal of the glycine linker fragment. The purpose of the linker was to generate intramolecular flexibility and reduce structural constraints (Reddy Chichili et al., 2013).

The recombinant cassettes, henceforth referred to as CTB (983 bp), CTB-TcpA (1,055 bp), and CTB-Ag85B (1,958 bp), were transfected into WT Binnatoa rice calli using the *Agrobacterium*-mediated transformation method. Genomic and complementary DNA revealed that the genes were both integrated into the genome and expressed in seeds, with no corresponding signals detected in wild-type control plants (Fig. 2). The introduced genes were inherited according to Mendelian laws and were repeatedly confirmed for transgenicity at consecutive generations to ensure stable maintenance and expression.

Immunoblot analysis and quantification of plant-expressed recombinant proteins

A Western blot analysis of seed extracts from several independent transgenic lines identified the presence of accumulated proteins with apparent monomeric mole-

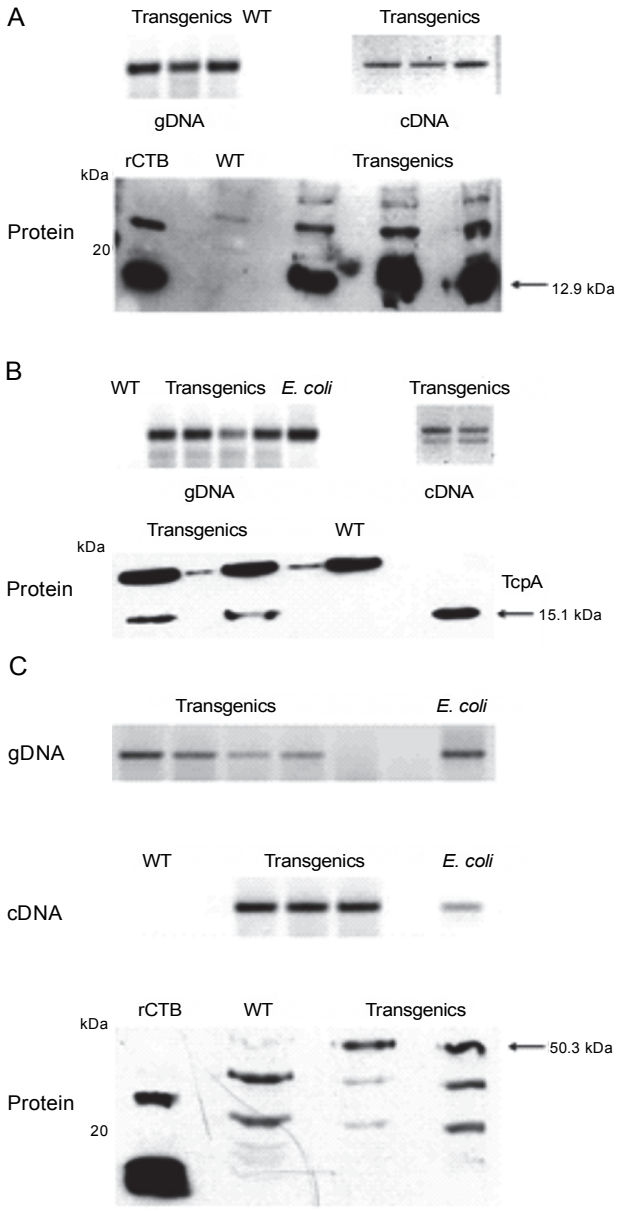

Fig. 2. Expression of fusion protein constructs in transgenic rice. The integration of the recombinant transgenes A) CTB, B) CTB-TcpA-P4, and C) CTB-Ag85B into genomic DNA (gDNA) was confirmed by PCR, while transcription was verified by the amplification of reverse-transcribed mRNA (cDNA); a Western blot analysis confirmed the expression of genes at the protein level as bands corresponding to 12.9 kDa, 15.1 kDa, and 50.3 kDa, respectively, for CTB, CTB-TcpA-P4, and CTB-Ag85 (panels labeled as proteins in A, B, and C) only in the transgenic lines; the protein bands are indicated by arrows; for the Western blots, antibodies against CTB and TcpA were used for blotting in A and B, respectively; in C, antibodies against rCTB were used, since no bands were observed with Ag85B-specific antibody; the CTB antibodies, however, detected the expected larger protein of ~50 kDa corresponding to the fused CTB-Ag85B; WT – wild type non-transgenic rice; *E. coli* – plasmid containing expression cassette; rCTB/TcpA – recombinant CTB and purified TcpA proteins

cular masses corresponding to the expected sizes of the chimeric proteins (12.9 kDa CTB, 15.1 kDa CTB-TcpA-P4, and 47.5 kDa CTB-Ag85B). Protein migration was slightly retarded in comparison to commercial rCTB protein produced in bacterial system (Fig. 2) and can be accounted for the presence of the hexa-peptide SEKDEL, failure of plant cells to remove the 2.9 kDa *GluB1* signal peptide, or by posttranslational modifications such as glycosylation (Mishra et al., 2006; Sharma et al., 2008). Total seed protein (TSP) from CTB-transgeic rice plants were analyzed in immunoblot using anti-CTB antibody which produced very clear CTB-specific bands (Fig. 2A). The fusion proteins (CTB-TcpA and CTB-Ag85B) were analyzed separately using both anti-CTB and either antigen-specific anti-TcpA or anti-Ag85B polyclonal antibodies for CTB-TcpA and CTB-Ag85B, respectively. For plants transformed with the CTB-TcpA construct, the rice-expressed protein was successfully recognized by both anti-CTB and anti-TcpA antibodies (Immunoblot shows result for anti-TcpA in Fig. 2B). Although monoclonal anti-CTB antibodies revealed a ~50 kDa protein in TSP isolated from CTB-Ag85B seeds, no such band was detected in the seed extracts when the Ag85B-specific antibody (Immunoblot shows result for anti-CTB in Fig. 2C) was used. No comparable bands with the antigens were observed in the extracts of WT rice. There was no difference in results even when non-denatured expressed proteins were subjected to the Western Blot analysis (results not shown).

Using a densitometry analysis with a known concentration of rCTB as standard, we found that the expression levels of CTB, CTB-TcpA, and CTB-Ag85B-detected using CTB-specific antibodies – represented up to 0.64%, 0.34%, and 0.02% of TSP, respectively. The lower accumulation of CTB in rice endosperm compared to previously reported (2.1%) may be due to the strain of rice used, as the same authors found a 6-fold discrepancy between the Kitaake and Hosetsu landraces (Nochi et al., 2007), whereas our analyzes revealed a 3-fold difference using our rice genotype, Binnatoa. In accordance with our observations, earlier studies have reported significantly lower expression levels of rice-derived recombinant proteins compared to unfused CTB (Matsumoto et al., 2009), possibly as a result of diminished stability or incorrect folding due to structural limitations and the inherently larger sizes of the chimeric proteins.

Proteins expressed in rice seeds are resistant to gastrointestinal degradation

To evaluate the capacity of our fusion proteins to withstand the effects of gastrointestinal degradation, TSP extracted from transgenic seeds were subjected to pepsin digestion as it is the main gastrointestinal digestive protease. Following an hour of *in vitro* digestion, a comparison of signal intensities between the untreated and degraded forms on a Western blot (Supplementary Fig. S1) revealed that ~75% of the accumulated CTB and CTB-TcpA-P4 proteins remained intact – a finding that is in accordance with the observations reported previously. (Nochi et al., 2007). These results indicate that bacterial proteins expressed in the protein bodies of transgenic rice seeds can largely be protected from enzymatic degradation within the digestive tract.

Binding of plant-derived CTB to GM1-ganglioside receptor

For the determination of CTB- and antigen-specific antibodies in orally immunized mice, ELISAs were performed using the serum of individual animals in each cohort. Increased levels of anti-CTB IgG (Fig. 3A) and IgA (Fig. 3B) responses were observed compared to mice immunized using WT rice powder. For IgG, responses peaked on day 40, with P values for serum IgG responses in mice immunized with transgenic CTB, CTB-TcpA-P4, and CTB-Ag85B corresponding to 0.008, 0.05, and 0.004, respectively. All three transgenic lines consistently elicited 3-fold increases in specific IgG responses over the WT. In the case of IgM, however, responses to CTB and CTB-Ag85B were the highest on day 40, with a relative lack of change in responses to CTB-TcpA. Noticeably, there was no higher response of IgM in any of the transgenic seed groups compared to that of the wild-type seed group. Furthermore, a higher trend (not statistically significant) of IgM responses to the wild-type rice compared to rCTB and CTB-TcpA transgenic rice was observed most probably due to the cross-reactivity (Supplementary Fig. S3). The immunized mice were seroconverted (i.e., at least 2-fold increase of antibody responses) for IgG and IgA by the third and fourth doses, respectively. The highest increase in IgA occurred on day 47 for the CTB antigen (~600-fold), with ~200-fold changes observed for CTB-TcpA and the lowest responses of ~50-fold generated by CTB-Ag85B. Lower IgA titers observed for the CTB fusions in contrast to the rCTB were probably due to differences in the uptake or influences of the plant matrix. Significant IgA responses in sera were detected on day 47 in the sera from mice immunized with transgenic CTB, CTB-TcpA-P4, and CTB-Ag85B (P = 0.01, 0.01, and 0.05, respectively), but were absent in the serum of mice fed with WT seed powder. This suggests that our chimeric proteins are biologically active and capable of folding into their native conformation after plant expression. Although there were drastic variations in the contents of CTB, CTB-TcpA, and CTB-Ag85B (0.64%, 0.34%, and 0.02% of TSP), the mice were not fed with the amount of powder containing equal amounts of transgenic proteins. Instead, they were fed with the highest possible amount of rice powder (120 mg/mouse) to maximize the immune responses as the optimal amount of protein required to effectively induce the immune response is unknown.

As observed with the immunoblots, the transgenic CTB-Ag85B rice-vaccinated serum did not detect the mycobacterial component of CTB-Ag85B by ELISA. Conversely, although the commercial anti-TcpA antibody functioned well in a Western blot analysis to detect a plant expressed CTB-TcpA, no detectable anti-TcpA serum responses were produced in TcpA-specific ELISAs. However, a dot blot analysis of the serum and BAL samples from mice immunized with transgenic rice seeds showed that the serum from 3 out of 6 (50%) mice fed with CTB-TcpA was positive to TcpA antigen, while 3 BAL samples (pooled) corresponded to serum samples that were found positive for TcpA antibody (Fig. 4). Although not all animals necessarily responded to immunizations at the same rate, the lack of antibody responses in some mice may indicate that a plant-expressed version of the P4 epitope of TcpA is only weakly immunogenic. The sera from mice that were positive in the dot blots, however, did not yield the same results in ELISAs. This sort of a differential response between ELISA and immunoblotting has been reported earlier, such as in the case of the CS6 antigen of ETEC, which binds to nitrocellulose membranes but not to polystyrene ELISA plates (Alam et al., 2014). Purified TcpA also failed to develop immune responses, suggesting that the oral immunization procedure may not have been adequate. In the absence of a bicarbonate buffer, the protein may have been subjected to enzymatic degradation in the acidic, proteolytic environment of the digestive system

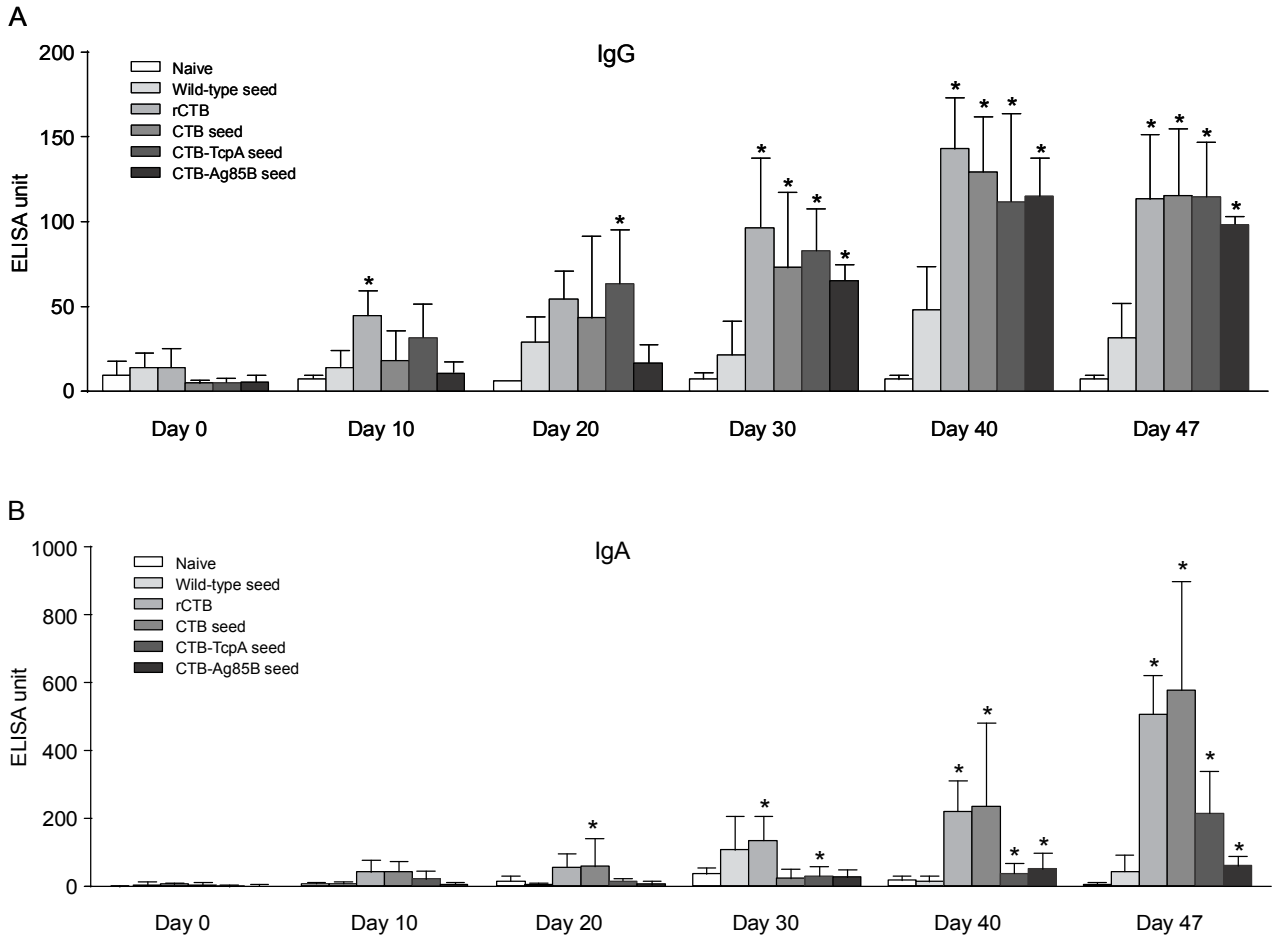

Fig. 3. CTB-specific antibody responses in the sera of immunized mice. ELISAs for the immunoglobulins A) IgG and B) IgA were performed for each cohort using the serum from an individual mouse, collected at six different time points; the columns indicate mean responses and the error bars represent standard errors of the mean; an asterisk denotes statistical significance ($P < 0.05$) compared to the non-transgenic WT control, n = 6-8

and was thus unable to stimulate a suitable immune response. The minimal responses observed using transgenic rice-expressed CTB-TcpA via dot blots can be accounted for by the encapsulation of the structure within seed protein bodies, thereby inhibiting the immunogenicity of the recombinant protein.

Rice-derived recombinant proteins induce immunity in the respiratory mucosa

In order to determine whether oral vaccination with transgenic rice can elicit immune responses, not only in the systemic compartment but also in the mucosal area, we performed CTB-specific ELISA using BAL fluid collected from the lungs of immunized mice. The incomplete communication between different mucosal compartments, possible transcompartmental trafficking of mucosal lymphocytes between the respiratory and gastrointestinal lamina propria, was reported already earlier (Kunisawa et al., 2008; Macpherson et al., 2008). As the mice were gastric-gavaged with solubilized seed powder prepared in a different room away from the mice room, there was no chance of inhaling of ground rice. Any spillover of the solution from the gastric passage to the aero-passage would have killed the animal right away. Therefore, all respiratory immune responses are assumed to have come through transcompartmental communication. IgA responses were observed in the CTB, CTB-TcpA, and CTB-Ag85B cohorts, with no response detected in the control group immunized with a wild-type Binnatoa seed powder (Fig. 5). Responses in all three cases were statistically significant, with CTB-only stimulating the highest fold-change of nearly ~200 above the baseline. P values for BAL IgA responses on day 47 in mice immunized with transgenic CTB, CTB-TcpA-P4, and CTB-Ag85B

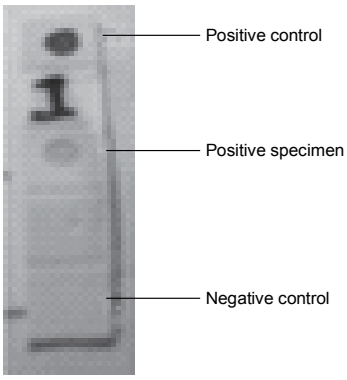

Fig. 4. TcpA specific antibody response in the serum and bronchoalveolar lavage (BAL) samples of mice immunized with transgenic rice seeds; an immunodot blot assay for the TcpA antigen was performed for each cohort using the serum and BAL sample from an individual mouse, collected at two different time points D0 and D47; the column strip represents the nitrocellulose membrane in which the upper circular dot indicates as positive control and the middle dot indicates a representative positive sample containing an antibody to TcpA and the lower portion of the blot paper indicates the negative control where no dot was observed

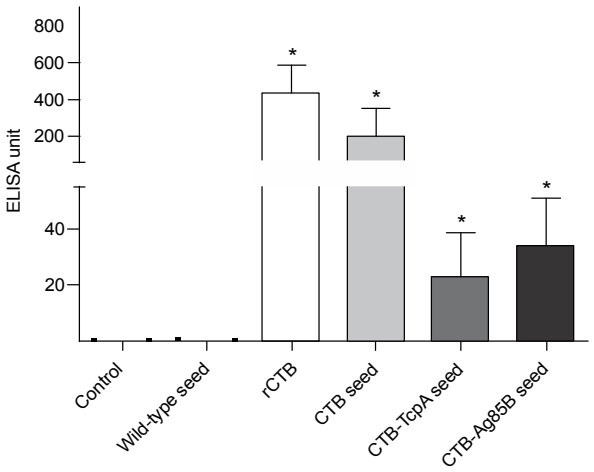

Fig. 5. IgA responses in the broncho-alveolar lavage (BAL) fluid of immunized mice; BAL fluid was retrieved on day 47 from the trachea of euthanized mice and used to carry out CTB-specific ELISAs; the columns indicate mean responses, and the error bars represent standard errors of the mean; an asterisk denotes statistical significance ($P < 0.05$) compared to the vehicle control, $n = 6$-8

were 0.004, 0.008, and 0.04, respectively. Due to low responses in TcpA-specific ELISAs, we conducted TcpA-specific dot blots using pooled BAL fluid from the mice that tested positive in sera. A positive response was elicited, with no signals in the wild-type or LPS controls, indicating that CTB-TcpA did in fact stimulate immune responses in the lung mucosa. Given the overall poor response of the anti-Ag85B antibody, no Ag85B-specific ELISAs were performed using BAL fluid.

CTB plays an inevitable role as adjuvant

The purpose of coupling CTB to the antigen was to provide an adjuvant effect on the antigen, which in this instance was dual. One was to provide an enhanced uptake of the antigen from the gastrointestinal tract through the interaction of the CTB with its receptor GM1 ganglioside expressed on the surface of the gut epithelial cells. The other effect was a crucial entry point of the antigen into the mucosal immune system in the case of oral vaccination (Moreno-Altamirano et al., 2007). Another very important role of CTB was to enhance the internalization and presentation of the antigen by major histocompatibility complex-II (MHC-II) through the interaction of CTB with GM1 present on the professional antigen presenting cells, e.g., macrophages, dendritic cells, and B-cells. The interaction of CTB with GM1 increases the expression of MHC-II molecule on the surface of B-cells. Therefore, the presentation of CTB and its coupled antigen are also vastly increased (Francis et al., 1992). Typically, only a few antigen-specific B-cells out of the total B-cell repertoire capture the uncoupled antigen through the interaction with antigen-specific B-cell receptor (BCR) and send the antigen to the MHC-II loading compartment. On the other hand, a CTB-coupled antigen is sent to the MHC-II loading compartment through the interaction of CTB with GM1 expressed almost on the entire B-cell population. The MHC-II-mediated presentation of an antigen on the B-cell surface is crucially important, because the antigen: MHC-II complex is recognized by the antigen-specific helper T-cells that have already differentiated in response to the same antigen. The helper T-cells produce cytokines that stimulate the differentiation and proliferation of antibody secreting and memory B-cells. Therefore, in comparison to the uncoupled antigen, CTB-coupled antigen stimulates a robust antibody response (Stratmann, 2015). Assuming this inevitable role of a coupling adjuvant, we did not feed the mice with the seed powder transgenic for uncoupled antigen (e.g., only TcpA or only Ag85B seed powder). As experimental evidence, we fed one group of mice with commercial TcpA antigen in PBS which resulted in responses similar to naive mice (data not shown).

Protein identification of CTB-Ag85B by mass spectrometry

The commercially purchased anti-Ag85B antibody used in our experiments consistently failed to bind with the CTB-Ag85B fusion protein, although anti-CTB antibodies indicate the presence of the expected 50 kDa protein in our transgenic rice. To determine whether any deleterious mutations had occurred, we sequenced the recombinant gene from our transgenic lines at the genomic (DNA) and transcriptomic (reverse-transcribed cDNA) levels. In both instances, the retrieved nucleotide sequences were identical to those of the original plasmid constructs, indicating that transgene integration and stability were unaffected at the genome and transcript levels. Additionally, the gene cassette design was eliminated as a potential source of error due to the effective recognition of the TcpA component of our CTB-TcpA-P4 fusion protein by an immunoblot analysis, given that the 2 constructs were identical in every aspect except for the sequence of the vaccine component itself.

For protein identification, we aimed to detect two main regions of the CTB-Ag85B protein – CTB and Ag85B – by mass spectrometry. Total soluble proteins extracted from transgenic rice seeds were separated on SDS-PAGE, and the ~50 kDa band region containing the target peptide was submitted to two different proteomics groups. To ensure reliability, one group was not provided with any additional details about the peptide, whereas the other was supplied with our full-length sequence of interest. The first group recognized strings of uninterrupted sequence of at least 20 amino acids of the Ag85 protein, since they were not given any clue about the protein. The other group which was provided with the identity of the protein showed 45% (212 of 468 amino acids) identity along the complete sequence. This indicates that both regions are present in our plant-based fusion protein (Supplementary Fig. S2).

The failure of the anti-Ag85B Ab to recognize CTB-Ag85B can be attributed to the fact that the antigen originally used to produce the antibody was sourced from bacterial cells. Ag85B contains at least 10 glycosylation sites. The potential glycosylation positions are 2, 5, 71, 134, 142, 160, 243, 253, 299, and 301. Among them 71, 243, 253, and 299 are N-glycosylation sites while 2, 5, 134, 142, 160, and 301 has O-glycosylation site (Supplementary Fig. S4) (Chauhan et al., 2013). The predicted glycosylated amino acids that match with the mass spectrometry studies are 71 for N-glycosylation and 2, 5, and 134 for S/T glycosylation (Supplementary Fig. S2). Due to these glycosylation sites, the production of Ag85B in plants may exert changes in the protein's posttranslational modification pattern and secondary structure, thereby preventing plant Ag85B from being recognized by antibodies against bacterial Ag85B. To consolidate this hypothesis, future experiments should involve attempts to enzymatically deglycosylate the fusion proteins prior to performing immuno-reactions or to produce the plant codon-optimized sequence in bacterial cells, in order to assess whether this facilitates detection.

Conclusion

We aimed at developing an oral delivery system for recombinant vaccines based on the expression and accumulation of fusion protein combinations that have not been produced in transgenic rice seeds earlier. Our findings demonstrate the potential of transgenic rice vaccines to express antigens to provide enteric and respiratory protection through the reinforcement of mucosal and systemic immunity. The weaker immunogenic response of the fusion proteins with CTB may indicate epitope-shielding, particularly in the case of the larger antigen CTB-Ag85B. Thus, our work highlighted the importance of rigorous screening of potential antigens via *in vivo* testing to determine whether they remain immunogenic under the proposed oral delivery system. Further testing with different combination of adjuvants and smaller epitopes can expand the scope for the development of edible vaccines strategies for the two common infectious agents, *V. cholerae* and *M. tuberculosis*. Further studies are required to provide new insights and also hopefully target other agents causing mucosal or systemic diseases of global concern in the future.

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Disclosure

None of the authors has any conflict of interest associated with this study.

Author contribution

Z.I.S., F.Q. designed the experiments. S.Z. cloned, developed, and confirmed the transgenic plants with some help from M.K.K., M.K.K., M.M.A., S.M.T.I. did the western blotting, immunization and sample collection, S.M.T.I., M.I.U., N.I.B., S.I., performed the immunological experiments, S.M.T.I., M.I.U., T.R.B. analyzed data, S.Z. wrote the manuscript with help from S.M.T.I., M.K.K., Z.I.S., and F.Q. All authors read and approved the manuscript.

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