ORIGINAL PAPER

Seed-Based System for Cost-Effective Production of Vaccine Against Chronic Respiratory Disease in Chickens

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Abstract
The production of vaccines in plant cells, termed plant-made pharmaceuticals or molecular farming, is a promising technology for scalable production. Compared to mammalian cell lines, like Chinese Hamster Ovary (CHO) or bacterial cells, plants can be grown with less cost on a large scale to make vaccines antigens and therapeutics affordable and accessible worldwide. An innovative application of this alternative system is the production of vaccines in edible tissues that can be consumed orally to deliver protein antigen without any further processing. In this project, we report stable expression of amino acid sequences corresponding to the TM-1 gene of *Mycoplasma gallisepticum* as a candidate vaccine antigen against Chronic Respiratory Disease (CRD) in chickens using wheat seed's tissues as a production host. Molecular and immunoblotting analysis confirmed the ubiquitous expression of a recombinant 41.8-kDa protein with an expression level of 1.03 mg/g dry weight in the endosperm tissues. When orally delivered, the plant-made vaccine was effective in terms of developing antibody response in animal model i.e., chicken without any detectable weight loss. Two doses of orally delivered plant-made TM-1 vaccine candidate elicited the immune response and protective effect against MG virus challenge at the level comparable to commercially available inactivated vaccine against CRD. Our study demonstrates that plant-made vaccines are not only safe but also scalable and cost-effective with prolonged stability at room temperature.

Abbreviations
CTB   Cholera toxin B subunit
MG   *Mycoplasma gallisepticum*
CRD   Chronic respiratory disease
PDs   Protein drugs

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Introduction

Plants can be an attractive system to produce immunogens to control different human and veterinary diseases [1]. The idea of utilizing plants as a bioreactor for the expression of veterinary vaccines has been studied extensively in the last decade [2]. Progress in plant genetic engineering has opened novel opportunities to use plant cells, cell culture, organs, and tissues as a bioreactor for a safe and cost-effective production of various vaccine antigens to mitigate infections. Plants provide an alternative expression system to microbes or mammalian cells grown in fermenters, which have disadvantages of limited capacity, higher production costs, and in the case of bacteria, a lack of post-translational modifications and requires a high initial investment. Moreover, molecular biopharming has an edge over other production systems for recombinant proteins in terms of risk of contamination with

human and animal pathogens, microbial toxins, or oncogenic sequences. In the last two decades, plants have been used as a viable and efficient bioreactor for the large-scale production of recombinant proteins including pharmaceutical proteins, antibodies [3], hormones, replacement enzymes [4], industrial enzymes [5] as well as vaccine antigens against infectious diseases [6]. Till now, a variety of vaccines against veterinary diseases have been expressed using plant tissues as bioreactors [7]. Infectious diseases directly or indirectly affect daily routine and businesses that result in huge economical losses. For instance, bovine spongiform encephalopathy known as mad cow disease resulted in huge economic losses of over 5.3 to Canadian beef and dairy industries [44]. Such gigantic losses were recorded two years after the identification of the first infected animal, describing the urgency of novel methods for vaccine production. The proof-of-concept for such studies has already been accomplished in previous studies and plants have been found as a suitable host for the expression of edible vaccines for Newcastle disease [8], influenza, Ebola, infectious bronchitis virus (IBV), infectious bursal disease virus (IBDV) in chickens [9]. The plant-made ZMapp antibodies against Ebola and influenza were not only encouraging but also paved a way for plants to be further utilized as a production host to combat infectious diseases in cost-effective manner [45]. Plant expressing vaccines against Newcastle disease virus was approved by USDA in 2006 [10] therefore, regulatory approval of plant-made vaccines against veterinary disease can be achieved following the same regulatory pathway.

To mitigate bacterial and viral diseases in animals, vaccination is the first line of defense to lower the cost of therapeutics and harmful effects of using antibiotics [11]. However, immunotherapeutics and vaccines are limited in supply despite its gigantic sales of animal health products of $23 billion in 2013. In addition, the cost of veterinary vaccines accounted for $5 billion [43] which contributes to the rising prices of protein diets. The most prevalent problem in poultry, especially at a larger scale setup, includes zoonotic diseases which are not only a threat to poultry producers but also are a great risk for poultry genetic resources [8]. Thus, suitable veterinary strategies against such diseases are required for a poultry setup. At present, there are occurrences of Chronic Respiratory Disease (CRD) worldwide and possess implacably drastic effects on small-scale poultry producers in underdeveloped countries. *Mycoplasma gallisepticum* (MG) causes respiratory disease primarily in chickens and turkeys, which can spread both vertically and horizontally, and can also infect gamebirds, pigeons, ducks, geese, peafowl, and wild birds. Symptoms of CRD include stress caused by moving the birds, de-beaking, operations handlings, or unfavorable conditions (bad ventilation or cold). At present, poultry management activities such as feeding and watering, treatment with herbs, antibiotics, vaccines, and cleaning poultry houses are mainly carried out against diseases [12]. The use of antibiotics is one of many important tools in the treatment of poultry diseases. The use of antibiotics has brought unintended impacts on poultry production industries, such as the increase of infections in animals and the decrease in animal production [13]. Also, antibiotic residues can remain in the body for a long time and may also greatly reduce scavenger wildlife populations. The potential relationship between circulating antibiotic residues and the presence of bacterial and fungal pathogens causing severe diseases. To overcome these problems, an innovative strategy is to develop a cost-effective vaccines against poultry diseases. Vaccination also enhances the immune response, without affecting the normal bacteria in the body and can easily be decomposed. Plant-based vaccines should also be encouraged as they can easily be manufactured and the production costs may be significantly lower as compared to conventional production systems.

Cost-effective and easy-to-administer vaccination is also a suitable alternative strategy to antibiotics used to mitigate or combat bacterial and viral diseases in animals. In this study, in view of mounting production costs for veterinary vaccines and exceeding usage of antibiotics, we expressed a candidate antigen vaccine against CRD in wheat seeds as a promising model for cost-effective vaccine development in plants that can be delivered orally without requiring any purification or downstream processing.

We demonstrate the feasibility of developing plant-based orally delivered vaccines that involve no downstream processing for protein recovery and purification. The oral delivery of vaccine candidates has been reported earlier in numerous studies [14–16]. The downstream processing involves expensive chromatographic resin and equipment that accounts for more than 80% of total production costs and can be eliminated if the vaccine is delivered orally without necessitating further processing [17]. The oral delivery of protein drugs (PDs) is facilitated by a plant cell wall that contains β-1,4 and β-1,6 linkages resistant to hydrolysis and allows PDs to be functionally active without any degradation during passing through enzymatic conditions in the stomach. Further, certain gut bacteria that have the ability to hydrolyze plant cell wall contribute to the release of PDs and their absorption into the bloodstream.

In this study, we genetically engineered a stable TM-1 protein as a promising candidate antigen in wheat seed tissues. The vaccine candidate was orally administered to chickens and produced antibodies those able to inhibit infections after the second drug dosage. Our findings demonstrate that oral delivery of vaccines is a promising approach that can revolutionize the current production and delivery system for safe and affordable vaccine development.

Materials and Methods

Construct Development and Wheat Transformation

The binary vector pPZP211 vector was purchased commercially (Creative Biogene). A rice endosperm-specific promoter *GluB-4* [18] and a Nos terminator (GenBank: AJ007623) were inserted upstream and downstream of the multiple cloning site (MCS) at *EcoR*I/*Kpn*I and *Xba*I/*Hind*III sites, respectively. The codon-optimized complete CDS of TM-1 protein of *Mycoplasma gallisepticum* (MG; GenBank: E09896) was followed by the endoplasmic reticulum-retention signal KDEL (Lys-Asp-Glu-Leu) linked to C-terminal of TM-1. The CTB (cholera toxin B subunit) coding sequence followed by a furin enzymatic cleavage site (RRKRSV) was inserted immediately upstream of TM-1. The entire PGluB-4-CTB-furin-TM1-KDEL-Tnos cassette was synthesized (GenScript, NJ, USA) and subcloned in pPZP211 at *Kpn*I/*Xba*I sites (Fig. 1A).

Wildtype wheat (*Triticum aestivum* L. cv Bobwhite) was used to generate transgenic plants in this study. PDS-1000/He particle gun or biolistics (Biorad, Germany) was utilized for direct bombardment of the plant tissue. Gold particles of size (0.6 μm) were coated with 20 μg plasmid DNA using 950 psi rupture disks for bombardment. Plants were grown in a greenhouse with 16 h daylight at 24 °C. The plants were grown in the greenhouse under a 16 °C day and 12 °C night for the first 40 days with a photoperiod of 10 h, followed by a 24 °C day and 18 °C night regime with a 16 h photoperiod till maturation. The lower temperatures helped to tiller and produce more seeds. The spikes were observed closely, and immature embryos were isolated 14 days after anthesis (size 0.5–1.5 mm) under aseptic conditions and were placed on induction media (MS media 4.3 g/L, Maltose 40 g/L, Glutamine 500 mg/L, 2, 4-D 2 mg/L, Gelrite 2.5 g/L and
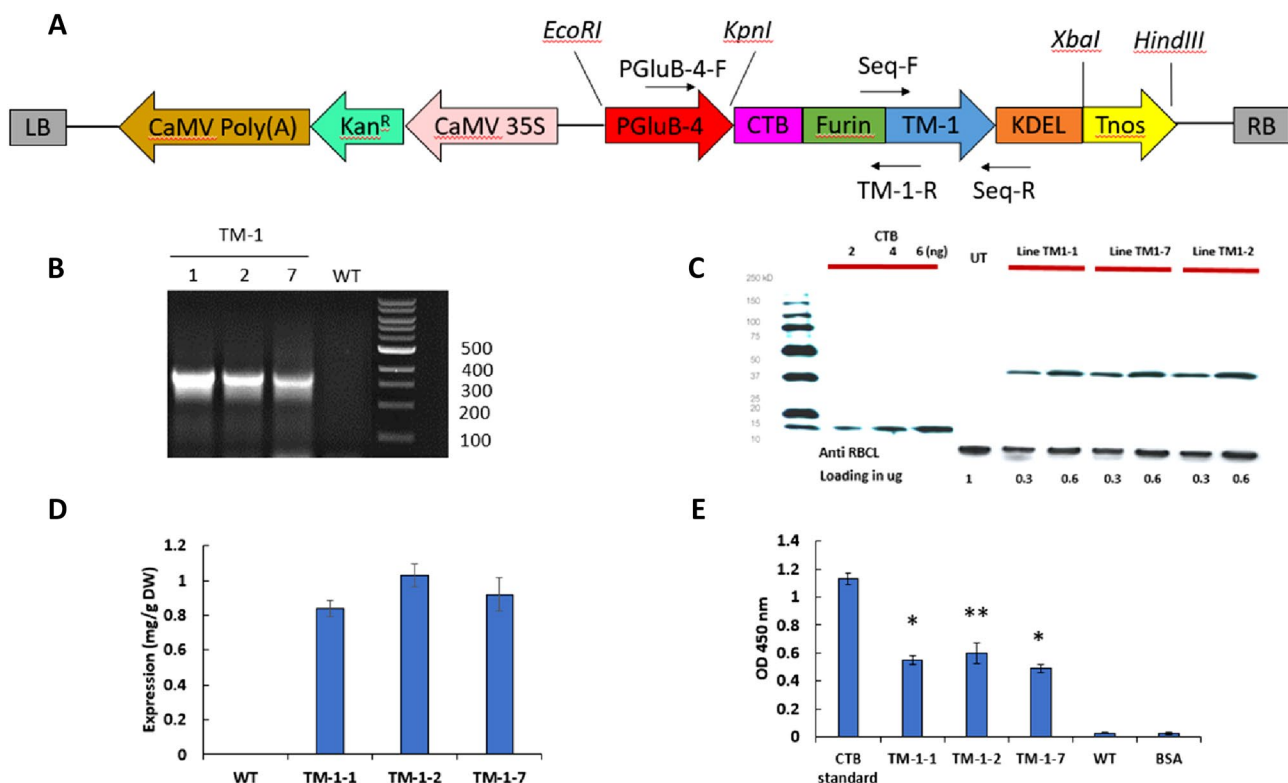
Fig. 1 A The schematic diagram of expression cassette of pPZP211: CTB-TM-1. Genetic components included are: PGluB-4, promoter of glutelin gene GluB-4; CTB, Cholera non-Toxin B subunit; Furin, Furin cleavage site; TM-1, TM-1 protein of *Mycoplasma gallisepticum* (MG); KDEL, Endoplasmic reticulum retention signal; Tnos, termination of the nopaline synthase gene from *Agrobacterium tumefaciens;* CaMV 35S, promoter of 35S RNA from *Cauliflower Mosaic Virus*; KanR, Kanamycin resistant gene; CaMV Poly(A), *Cauliflower Mosaic Virus* polyadenylation signal; LB and RB, left and right border of T-DNA repeat, respectively. Arrows indicate PCR and sequencing primers. **B** characterization of genomic integration of pPZP211: CTB-TM-1 cassette in different transgenic lines by PCR using primers (CTB-F and TM-1-R). **C** Validation of recombinant CTB-TM-1 expression in three lines by Western blot with anti-CTB antibodies. **D** quantification of recombinant CTB-TM-1 expression in C. **E** GM1 ELISA detecting the folding of CTB-TM-1 and its binding with GM1 receptor. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$

adjusted pH 5.8) for 5–6 days in the dark at 26 °C before bombardment for callus induction.

Total DNA was extracted from wheat seeds for confirmation of genetic integration of PGluB-4-TM-1-Tnos construct in wheat seed genome by Polymerase Chain Reaction (PCR) and sequencing with primers targeting transgene TM-1 (Seq forward: 5′- agcattgggatttctagctg -3′; Seq reverse: 5′- agttgaagcatacaaagcac -3′; Fig. 1A).

Regeneration and Selection

The transgenic plants were grown in soil to obtain T_1 wheat seeds. To confirm the transgenic nature of the plants, the T_1 seeds were soaked overnight in distilled water and the embryos were removed aseptically. The excised embryos were placed on MS media (sigma M5519) half-strength with 50 mg/L of kanamycin for selection.

Nucleic Acid Isolation and PCR Confirmation

Plant genomic DNA was extracted from 100 mg plant material i.e., seeds using the CTAB (cetyltrimethylammonium bromide) method using DNA extraction buffer 1% (w/v) SDS, 100 mM NaCl, 100 mM Tris base, 100 mM Na2EDTA, pH 8.5 by HCl), as described earlier by Murray et al. (1980). PCR Primers (TM-1 forward: 5′- agcattgggatttctagctg -3′; TM-1 reverse: agttgaagcatacaaagcac was utilized to confirm the transgene integration. The PCR analysis was conducted for the presence of DNA encoding vaccine antigen. Specific primers to the promoter region and TM-1 gene amplified the sequences in transgenic wheat seed tissues in accordance with the estimated 330 bp of fragment. The DNA extracted from transgenic wheat seeds was used as a template for PCR analysis. The amplification (25 µl total volume) with 100 ng of template, for TM-1, was carried out at 94 °C for 5 min, 94 °C for 1 min, 56 °C for 1.5 min, and 72 °C for 1 min, with a total of 35 cycles, and final extension of 72 °C for 5 min. The amplified PCR product was resolved on a 1% agarose (w/v) gel electrophoresis and was finally analyzed under UV light to confirm the presence of the desired amplicon of expected size. After confirmation, all the positive plants were self-pollinated to obtain T_1 generation and were utilized for all analysis.

Recombinant Protein Extraction

To find out the accumulation of recombinant fusion proteins using a vibrator 50 mg of seeds were powdered. The samples were homogenized with 1:3 ratio of protein extraction buffer (3% (w/v) CTAB, 1.4 M NaCl, 0.2% (v/v) β-Mercaptoethanol, 20 mM EDTA, pH 8.0, 100 mM Tris–HCl, pH 8.0, 1% (w/v) PVP40). The samples were placed at 4 °C with continuous vibration for 30 min. The total soluble protein (TSP) concentration of the extracts was determined by Bradford assay using Bradford protocol 19 with bovine serum albumin (BSA) as standard, and TSP from wild type (WT) plant as a negative control.

SDS-PAGE and Western Blot Analysis

The total protein extracted from transgene positive wheat seeds tissues was then separated by SDS-PAGE based on their molecular masses at 90 V for 50 min. The expression of vaccine antigen in seed tissues was verified by western blot analysis. The band was separated using 8% gel and was incubated with PBST containing 5% skimmed milk (w/v) for one hour with continous shaking. The blot was then incubated in a dilution of 1:10,000 with polyclonal rabbit anti-CTB antibody (Sigma–Aldrich, Inc,) overnight at 4 °C with continuous shaking. The blot was then washed three times with PBST and then was incubated with a secondary goat anti-rabbit IgG- HRP antibody (Southern Biotechnology, Birmingham, AL) with a dilution of 1:5000 in PBST containing 5% skimmed milk. The expected bands corresponding to CTB-TM-1 fusion protein were detected by NBT/BCIP and developed with X-Ray with 5 s exposure time. The quantitation of a specific band was performed using the software ImageJ.

ELISA and GM1 Binding Assay

For detection of anti-MG antibody titer in immunized chickens, chickens were bled, and the serum was analyzed by the ELISA following the manufacturer's instructions (Bio-Chek USA). All the samples were analyzed in triplicate. For the GM1 binding assay, the 96-well plate was coated with 100 µL GM1 ganglioside receptor overnight at 4 °C. Then the plate was washed with 250 µL PBS-T and blocked in 1% casein blocking solution (100 µL) for 1 h at 37 °C. After washing thrice in PBS-T, plant or serum samples incubate in different dilutions in the plate for 1 h at RT. The plate was washed 3× for 5 min each with PBS-T and was then added 50 µL of primary antibody (polyclonal rabbit anti-CTB antibody for plant sample and anti-MG antibody for serum sample) and incubated for 1 h at 37 °C, with continuous shaking. The plate was then washed three times for 5 min each with PBS-T (250 µL) and added secondary antibody (goat anti-rabbit IgG- HRP antibody) in 1% casein solution for 1 h at 37 °C with continuous shaking. The plate is finally washed three times for five min. The reactions were developed by adding 100 µL of microplate colorimetric TMB substrate to each well and then incubated for 10 min or until the color had developed. The reaction was stopped by adding 100 µL

of 1 N HCl. The reading was recorded using a SpectraMax spectrophotometer at 450 nm.

Immunization and Challenge Procedure

The main objective of the study is to orally deliver the seeds expressing vaccine antigen. We included 100 chicks (20 days old) that were separated randomly into four groups of 25 birds each. Three groups were orally delivered PBS-premixed seeds powder containing 15 μg recombinant TM-1 protein (estimated on basis of total active protein expression), the same amount of WT seed powder (in grams), and PBS (Phosphate-buffered saline) buffer twice on day 7 and 21. The fourth group was inoculated with MG attenuated vaccine (Vaxsafe® MG, Strain ts-11, Biproperties Pvt. Ltd, 8×10^6 CFU) via the ocular method. MG virus S6 strain (5×10^6 CFU) challenge was conducted through eye route for all groups at weeks 4 and 5. Clinical parameters were recorded daily after the challenge with MG virus strain as described previously for 2 weeks [20]. Dead chickens before termination of the study were necropsied. All others were euthanized and examined 2 weeks after the MG challenge. Briefly, the main clinical scores consisted of four levels in terms of symptomatic severity as follows: 0—no symptom; 1—clear nasal exudate; 2—cloudy nasal exudate; 3—turbid nasal exudate with foamy eyes and/or swollen infra-orbital sinuses. For air sac lesions, scores were observed and examined at 14 days after the challenge as follows [21]: 0—no lesions; 1—mild lesions; 2—moderate lesions; and 3—severe lesions. Individual bodyweight was also recorded every week. All experiment groups were challenged with the MG virulent strain (5×10^6 CFU) one week after the second immunization by ocular delivery, followed by recording clinical parameters for additional 2 weeks.

Fertility Rate Quantitation

The Fertility rate analysis was conducted following an earlier published method (Mavi et al. 2020). Briefly, after immunization, no more than 50 eggs were collected from each hen in all groups and incubated at 37 °C for 21 days. The number of eggs was counted, and fertile eggs were determined by candling on the 21st day for embryo development. The fertility rate was calculated as a total number of fertile eggs divided by the total number of eggs collected and multiplicated with 100%.

Statistical Analysis

The statistical analysis of this study was carried out using the one-way ANOVA analysis and indicated by *P*-value.

Results

Creation and Characterization of Transgenic Wheat Seeds Expressing Recombinant TM-1

Lower expression of plant-made biopharmaceuticals is a great concern and hurdle for its commercialization. Several efforts including enhanced promoters, regulatory elements, protein targeting, utilization of various plants and their tissues, and codon optimization were made to improve overall yield. It has been known that mRNA stability and translational efficiency are enhanced by codon optimization due to idealized tRNA usage preference of recombinant TM-1 biosynthesis in host cells [22]. Therefore, the coding sequence of TM-1 was optimized during which 88 out of 262 codons in the native sequence were replaced for expression, resulting in an elevated GC% from 31.93 to 46.44% (Supplementary Fig. S1). The rice seed-specific promoter *GluB-4* was included in the construct as it can drive and target the recombinant protein in rice endosperm with the highest translational activity among the other 14 rice seed-specific promoters [18] (Fig. 1A). The ER retention signal peptide KDEL linked to the 3′ end of the TM-1 coding sequence has been shown in different studies to effectively increase recombinant protein expression in rice endosperm [23]. CTB fused immediately upstream of TM-1 allows transport of TM-1 through gastrointestinal epithelia to the circulation system by binding to a mucosal membrane receptor ganglioside GM1 [22]. To achieve efficient delivery, furin cleavage sites are digested by furin, a ubiquitous protease in human cells, enabling the release of TM-1 after transmucosal transfer carried by CTB [24].

Three independent transgenic wheat lines were generated with particle bombardment and subsequent regeneration and selection. After screening, the line with the highest expression (based on western blot analysis) was selected for further analysis. To verify the insertion of CTB-TM-1 construct in the wheat genome, PCR with construct-specific primers was performed using genomic DNA from wheat T_1 transgenic endosperm (Fig. 1B). The presence of the CTB-TM-1 construct was detected in all three lines (Fig. 1B). The Western blot further confirmed the presence of recombinant TM-1 in wheat seed tissues from all lines with the mean expression level of 0.84 ± 0.046, 1.03 ± 0.068, and 0.92 ± 0.096 mg/g dry weight (DW), respectively, and correct size as expected (~ 41 kDa) (Fig. 1C, D). To evaluate the proper formation of CTB that binds to GM1 receptor, a GM1 ELISA binding assay was carried out with crude extracts (TSP) of wheat seeds tissues expressing recombinant CTB-TM-1. The ELISA results showed that CTB-TM-1 correctly binds

to GM1 in vitro when compared to CTB standard, and WT samples (Fig. 1E). In addition, unless otherwise indicated, the T_1 seeds of line TM-1–2 were used for all downstream analysis and processes due to their highest TM-1 expression level and prominent affinity to the GM1 receptor.

The transgenic nature of wheat plants transformed with the CTB-TM-1 cassette by biolistics were tested on MS $^1/_2$ media containing an antibiotic as a selection marker. All the embryos excised from PCR confirmed transgenic T_0 plants showed efficient regeneration as compared to wild-type embryos as a negative control. A total of 100 embryos excised from the transgenic wheat line were confirmed by transgene integration and showed regeneration (Fig. 2).

Stability, Functionality, and an Immune Response is Preserved After Storage for 6 Months

Plant seeds have the natural ability to protect the recombinant proteins such as vaccine antigen by encapsulation in the plant tissues, and therefore, allow its storage at ambient temperature, moisture, and light conditions, and thus simplifying its production and distribution. We have tested our plant-made vaccine antigen for confirmation of immunogenicity after long-term storage. The immunoblot (Fig. 3) confirmed TM-1 vaccine antigen can be stored at ambient temperature
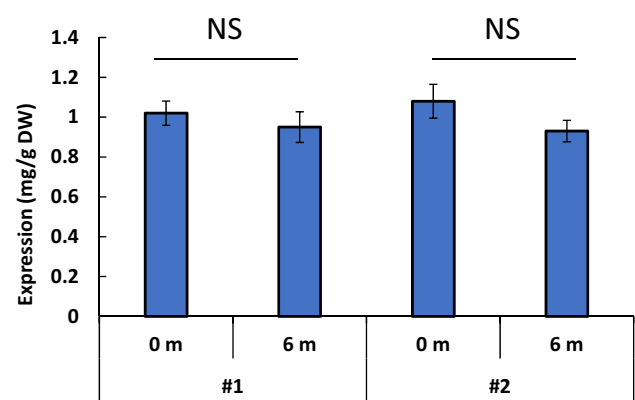
Fig. 3 Stability of recombinant CTB-TM-1 expressed in wheat seeds after long-term storage at ambient temperature (transgenic line: TM-1–2). The recombinant CTB-TM-1 was extracted from seeds powder stored immediately after harvest and 6 months later as described in method and the expression level was quantified by western blot with anti-CTB antibody

without degradation for at least 6 months. The confirmation of immune response is evidence of such bio-encapsulation where vaccine antigen was intact during passing through the digestive system. Cell wall-mediated bio-encapsulation allows proteins to withstand enzymatic digestion and can
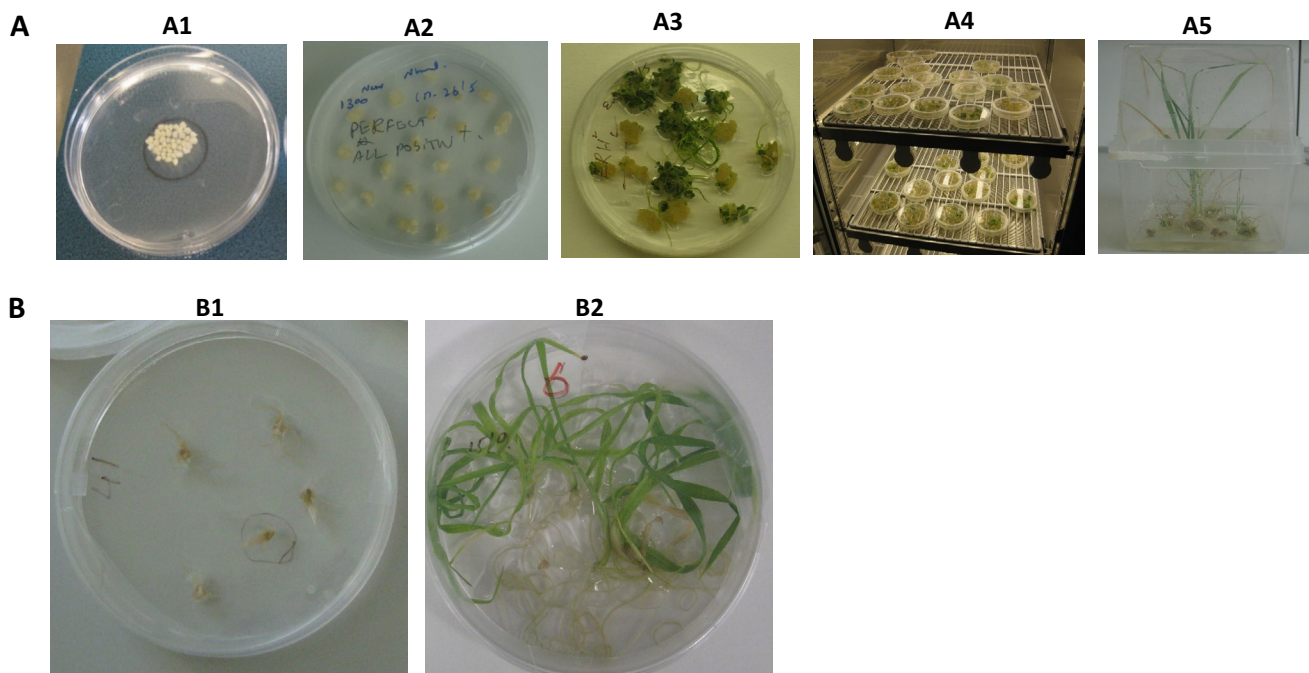
Fig. 2 Process of developing transgenic plant using Biolistics: Segregation of transgenic wheat lines: **A1** Immature embryos after bombardment with plasmid carrying TM-1. **A2** Callus development under dark with antibiotic selection. **A3** Plant regeneration in media containing selection marker. **A4** Plant regeneration and rooting in the growth chamber. *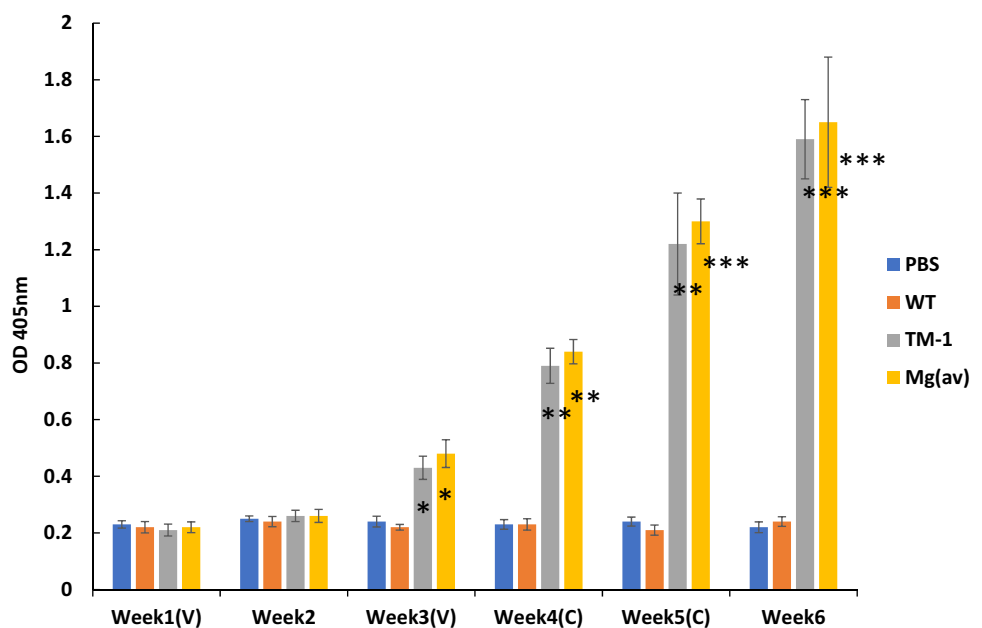*A5** Wheat plant expressing TM-1 vaccine candidate in rooting media, before moving to soil for growth. **B** Embryo rescue analysis. Wheat embryos excised from WT and positive plants and their germination on selection media. **B1** Wild type BobWhite as negative control. **B2** Growth TM-1 positive embryo excised from seeds after 10 days on MS half media as control for the experiment

reach the small intestine, where in this case the TM-1 vaccine antigen is incorporated into M cells in the follicle-associated epithelium (FAE), which finally results in a systemic immune response. CTB as a fusion protein is FDA-approved for the delivery of biopharmaceuticals into the immune system. The furin sites are incorporated between CTB and TM1 antigen for its delivery upon its cleavage. In addition, such results are encouraging because the protection to withstand enzymatic environments within the plant cell by bioencapsulation together with its stability at temperature of the storage and distribution. Here, we demonstrate that by employing both CTB fusion and furin cleavage plant-made vaccines not only have the ability to be successfully orally delivered but are also effective in animals.

## Immune Response to Vaccination of Recombinant TM-1

To evaluate the humoral immune response to vaccination of recombinant TM-1, serum from vaccinated chickens was analyzed with ELISA against anti-MG antibody starting from the first week after the initial vaccination. The titer of anti-MG antibody started to increase in TM-1 and MG attenuated vaccine groups one week after initial immunization, although not significantly higher compared to control groups, until 2 weeks later (Fig. 4). Moreover, while MG attenuated vaccine caused higher anti-MG antibody titer, it is not significantly different from the recombinant TM-1 group (Fig. 4). Notably, the amount of anti-MG antibody was improved even one more week after the second vaccination, leading to a greater significance compared to the control group vaccinated with WT and PBS buffer (Fig. 4).

Immunization of Recombinant TM-1 Protected Chicken Against MG Infection

We next tested if twice vaccination could adequately protect chickens from MG infection by monitoring changes in clinical symptoms and bodyweight after MG virulent strain inoculation. Two weeks after the challenge, the mean severity-based clinical scores in the control and WT groups were 2.73 ± 0.28 and 2.65 ± 0.26, which were significantly higher than in groups vaccinated with recombinant TM-1 and MG attenuated vaccine with a score of 0.72 ± 0.13 and 0.58 ± 0.19, respectively (Fig. 5), indicating an efficient systemic protection against MG virus infection. Additionally, bodyweight remained similar among all groups up to week
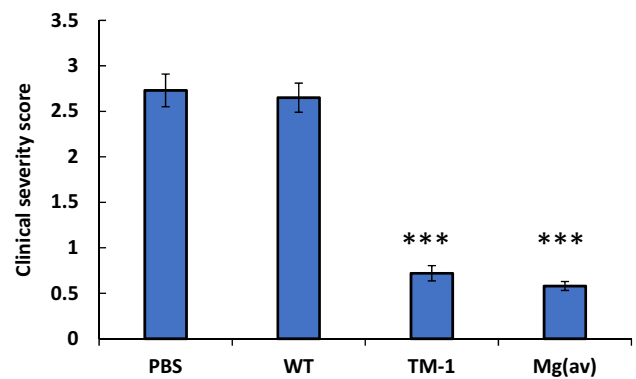
Fig. 5 Protective effect of recombinant TM-1 immunization against MG challenge. Clinical severity was observed and recorded for 2 weeks after MG challenge. Score categories were assigned according to the description in the materials and methods section in the text. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

Fig. 4 Immune responses to recombinant TM-1 vaccine. Anti-MG antibodies in chicken serum were quantified by ELISA after oral delivery of recombinant TM-1 vaccine, WT, and PBS except for the group inoculated with attenuated MG vaccine [MG(av)] by injection. (V) and (C) represent the week when vaccination or challenge occurred, respectively. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

5, suggesting vaccination of recombinant TM-1 antigen and attenuated MG vaccine did not affect gaining weight (Table 1). Nevertheless, chickens in TM-1 and attenuated MG vaccine groups gained significantly more weight than those in the control group one week after the MG virus challenge (Table 1), confirming the protective effect of recombinant TM-1 antigen prior to MG infection. Scores of air sac lesions showed a similar pattern. After 2 weeks of MG virus challenge, the presence of lesions was found significantly more in control groups compared to the other two groups, suggesting the recombinant TM-1 vaccine specifically protects air sacs in the avian respiratory system (Table 2).

Clinical Signs

In all groups, no adversary vaccine reaction was observed at the administration site. Also, there were no clinical symptoms, apparent phenotype alteration, or mortalities recorded in any bird after challenge exposure that also including all non-vaccinated controls. The birds were also monitored for their fertility; however, no fertility issues were observed during this study.

Discussion

In chickens, *Mycoplasma gallisepticum* is the primary etiological agent of CRD. MG is a major respiratory pathogen of poultry that causes a severe inflammatory response in the trachea of its host [25]. The pathogen leads to develop clinical symptoms such as ocular, sneezing, coughing, etc., and in severe cases causes pneumonia, bronchitis, and airsacculitis in chickens and turkeys [26]. After infection, MG resides on the mucosal surface causing epithelial cell degeneration, and also results in inflammatory cell infiltration into the mucosa. The infectious disease affects the poultry industry by drastically reducing productivity and accounts for huge financial losses. Besides lower production, commercially available vaccines to prevent and control outbreaks of the disease also add to the overall expenditures. MG infection control is achieved by maintaining a disease-free breeding

Table 2 Air sac lesion scores

Group	Chicken number (N)	Air sac lesion scores
PBS	25	2.6 (0.3)[a]
WT	25	2.5 (0.3)[a]
TM-1	25	0.7 (0.2)[b]
MG attenuated vaccine	25	0.5 (0.1)[b]

The superscript letters (a, b) represent date from groups that were statistically significant ($P < 0.05$) scales when compared to each other

flock [27] by means of antibiotics and both inactive and live attenuated vaccines. Mycoplasmas are generally resistant to beta-lactam antibiotics such as cephalosporin and penicillin due to the absence of a cell wall. However, mycoplasmas are sensitive to macrolides, tiamulin, tetracycline, etc., which, however, are very expensive. The commercially available vaccine such as strain 6/85 [28] F strain [27] and ts-11are applied for disease control. However, they are expensive and require maintaining a cold chain for storage and distribution. The commercially available inactive and live attenuated vaccines as well as recombinant live poxvirus (rFP-MG) are effective in controlling *Mycoplasma gallisepticum* outbreaks. Inactivated MG vaccines adjuvanted with aluminum hydroxide or aqueous oil emulsion are used in birds by subcutaneous or intramuscular routes and are also effective. However, they too are expensive, require maintenance of a cold chain, have to be administered to individual birds, and may cause local vaccine reactions. Alternatively, vaccine antigens expressed in edible tissues can reduce overall costs, require no application to individual birds or subcutaneous administration, can be stored at room temperature, and require no cold chain for transportation, hence, are best suited for poultry farms in rural settings. The present study confirmed that the plant-based recombinant TM-1 vaccine administered by feeding was as effective as a conventional attenuated vaccine applied intraocularly by eye drop (Fig. 4).

The availability of vaccines for chronic respiratory disease in chickens is limited and expensive. Plants that have the natural ability to store numerous proteins in seed tissues are an excellent destination to produce recombinant proteins

Table 1 Weekly bodyweight change

Group	Bodyweight (kg)						
	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
PBS	0.09 ± 0.01^a	0.15 ± 0.01^a	0.34 ± 0.03^a	0.64 ± 0.03^a	0.96 ± 0.06^a	1.55 ± 0.12^a	2.00 ± 0.11^a
WT	0.09 ± 0.01^a	0.16 ± 0.01^a	0.35 ± 0.03^a	0.66 ± 0.03^a	0.96 ± 0.07^a	1.54 ± 0.09^a	2.01 ± 0.13^a
TM-1	0.09 ± 0.01^a	0.16 ± 0.01^a	0.35 ± 0.02^a	0.64 ± 0.04^a	0.94 ± 0.06^a	1.57 ± 0.10^a	2.18 ± 0.14^b
MG attenuated vaccine	0.09 ± 0.01^a	0.15 ± 0.01^a	0.36 ± 0.02^a	0.65 ± 0.04^a	0.97 ± 0.08^a	1.59 ± 0.10^a	2.21 ± 0.15^b

The superscript letters (a, b) represent date from groups that were in statistically significant ($P < 0.05$) scales when compared to each other

[29]. Numerous proteins have been targeted in seed tissue, where the target proteins showed stability as well as functionality several months after storage at ambient temperature [30]. These plant-expressed vaccine candidates motivated this study to express vaccine candidates in plants targeted against CRD in chickens in a cost-effective manner.

In addition, supplementing chickens with numerous antibiotics to control different infectious diseases becomes more and more threatening. Residues of such antibodies have been found in chickens which are directly consumed by humans. Therefore, the promotion of alternative production systems for cost-effective vaccine development is required to ensure access to modern medicine and improve health, particularly in the so-called developing world.

In this study, as a prerequisite for oral delivery of the vaccine a seed-specific promoter was used to induce TM-1 expression in seed tissues. The stable transformation of the transgene carrying expression cassette into developing wheat calluses was achieved by biolistics. Expression of the TM-1 protein is controlled by the rice endosperm-specific promoter *GluB-4* with focus on seed tissues. Although, the fusion protein was also detected with significanlty lower expression in vegetative tissues, this data was not included in this manuscript, since the aim of this study was to establish a seed-based vaccine for oral delivery. The seed-based system is favored because such tissues are natural production and accumulation tissues for numerous proteins. Taking advantage of such ability we targeted our candidate vaccine antigen to achieve higher expression and its bio-encapsulation. Seeds are relatively lower in water (10–40%) in comparison to other plant tissues where water contents are up to 90% of their wet weight [31]. Thus, plant seeds are an ideal vehicle with lower water content with denser proteins concentration which favors oral delivery of vaccines. Taking advantage of seed tissues, several vaccine antigens and antibodies showed prolonged stability for years without loss of activity under normal room conditions [31, 32].

Our results demonstrate the oral delivery for PDs is as a novel method for developing edible vaccines and has been reported earlier in several studies [14–16, 33–37]. The plant-made antigen we developed has the ability to withstand environmental conditions and can be stable at ambient temperatures for a longer duration. In general, the cold chain requirements for storage and delivery of conventional vaccines are major concerns to equitably vaccinate the population in developing countries where the average daily income is less than $2 [46]. Children die especially in developing world because of the unavailability of vaccines against life-threatening infectious diseases. In this scenario, the availability of vaccines targeting veterinary diseases is highly unlikely. Maintaining a cold chain without continuous electricity supply for its storage and distribution is near to impossible. Likewise, the poultry industry is heavily affected by zoonotic diseases where high cost and unavailability of vaccines are major concerns, and those available are costly and require cold temperature.

In this study, we demonstrate the oral route for vaccine delivery which is a promising alternative by drastically reducing the costs of production, since no further purification steps are required. Due to the plant cell wall's natural ability of bioencapsulation, the expressed antigen is protected against enzymatic degradation and preserving its immunogenicity and functionality in the gastrointestinal tract. A unique characteristic of plant cells is their ability to withstand the digestive enzymes present in the gastric system of humans and animals. Certain bacteria in the small intestine have the ability to break down the plant cell wall thereby releasing the vaccine which is subsequently absorbed [46]. The successful oral delivery of recombinant TM-1 antigen produced in wheat seeds was correlated by results showing dramatically increased anti-MG antibody titer in chicken sera after vaccination compared to those vaccinated with WT and PBS buffer (Fig. 4). This was further supported by the observation that oral vaccination of recombinant TM-1 antigen efficiently protected chickens from MG infection, while chickens in control groups were greatly infected and developed corresponding clinical symptoms (Fig. 5; Table 2).

Plant seeds have a naturally evolved system to express and accumulate storage proteins in large amounts of up to 40% of their weight [30]. The accumulation of such a large number of proteins distinguish seeds from green tissues and tubers with lower protein expression levels [38]. Protein stability in seed tissues for a longer duration at ambient temperature is a huge advantage where antibodies and vaccine antigens expressed in higher amounts may remain functional and stable for several years at room temperature [30, 39, 40]. Our recombinant antigen showed high stability at room temperature. This can be attributed to the generally lower water content in seeds that may help to prevent protein degradation under such conditions. Also, even though the protein concentration is higher in seeds than in leaves, the efficiency of harvesting may be lower. Furthermore, pathogens may mutate from time to time. Therefore, transgenic lines may be screened for effectiveness against upcoming mutated pathogens. If required, new transgenic lines can easily be developed since generating transgenic wheat is well established using both direct and indirect methods of transformation.

To date, there are only few plant-made vaccines and monoclonal antibody candidates that received regulatory authorization compared to standard microbial-based systems. Plant-made products, i.e., a poultry vaccine against NDV and an antibody against HBsAg are the front runners in a 30 year long down the road journey of molecular farming. The NDV vaccine is produced in a suspension culture of tobacco cells. However, there is no oral product so far on the market for

orally delivered plant-made vaccines [41]. Oral delivery of plant-made vaccine antigens received publicity and attention for their ease of delivery and cost-effectiveness. This study is a precedent for oral delivery of vaccine antigens targeted against animal diseases in seed-based tissue showing excellent expression efficacy, immunogenicity, and stability.

Concluding Remarks

This study presents proof of concept to produce an edible vaccine in wheat seeds targeted against Chronic Respiratory Disease (CRD) in chicken. The recombinant CTB-TM-1 protein of *Mycoplasma gallisepticum* (MG) expressed in transgenic wheat seeds remained stable and functional after long-term storage at ambient temperature. In addition, oral delivery of CTB-TM-1 vaccine bioencapsulated in wheat seeds successfully induced immune responses and protection against MG infection in chicken without the sacrifice of a healthy development. Thus, this study demonstrates the feasibility, simplicity, and efficiency of seed-bioencapsulated oral vaccines in treating zoonotic infectious diseases.

Declarations

Conflict of interest The authors declare no commercial or financial conflict of interest.

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