Antibodies induced by oral immunization of mice with a recombinant protein produced in tobacco plants harboring *Bordetella pertussis* epitopes

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Abstract

Bordetella pertussis causes whooping cough or pertussis, disease that has not been eradicated and is reemerging despite the availability and massive application for decades of vaccines, such as Boostrix® which is an acellular vaccine harboring two regions of S1 subunit of the pertussis toxin, one region of filamentous hemagglutinin and one region of pertactin. In 2008, the World Health Organization estimated 16 million new cases and 95% occurred in developing countries with 195,000 children's deaths. We attempt to improve the vaccine against whooping cough and reduce its production costs by obtaining plants and bacteria expressing a heterologous protein harboring pertactin, pertussis toxin, and filamentous hemagglutinin epitopes from *B. pertussis* and assessing its immunogenicity after oral administration to mice. First, we designed a synthetic gene that encodes a multiepitope, then it was cloned into a vector for transient transformation by infiltration of tobacco plants with low amounts of nicotine; the codon bias-optimized construct was also cloned into an *Escherichia coli* expression vector. Recombinant proteins from *E. coli* cells (PTF) and tobacco leaves (PTF-M3') were purified by nickel affinity with a yield of 0.740 mg of recombinant protein per g dry weight. Purified recombinant proteins were administered orally to groups of Balb/c mice using the Boostrix® vaccine and vehicle (PBS) as positive and negative controls, respectively. A higher mucosal and systemic antibody responses were obtained in mice receiving the PTF and PTF-M3' proteins than Boostrix® or PBS. These findings prove the concept that oral administration of multiepitope recombinant proteins expressed in plants may be a potential edible vaccine.

Key message

We demonstrated the biological activity of a multiepitope recombinant protein of pertussis produced in plants by the immunization of mice, as a proof of concept for the development of an oral vaccine.

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Introduction

Bordetella pertussis, is a Gram-negative aerobic, coccobacillus human pathogen that infects the epithelial cells of the respiratory tract. This pathogen is the etiological agent of whooping cough (also called pertussis), a severe respiratory disease that is the fifth leading cause of vaccine-preventable death in children under five years. Before 1950s, when pertussis vaccine was introduced to the market, pertussis was a major cause of pediatric mortality (Moreno et al. 2013; Ulloa-Gutiérrez et al. 2011). Also, during the last two decades, pertussis was also recognized as an important pathogen in adults (Kilgore et al. 2016). Despite that the World Health Organization has estimates that triple doses of pertussis vaccine have a coverage around 85%, this disease remains uncontrollable. Both in low-income and in many developed countries, pertussis remains prevalent and rise, but the apparent increase has not been clearly established although they include potential changes in surveillance systems, increased awareness and better diagnostic tools (Mooi et al. 2009; Locht 2016). Each year around 40 million new pertussis cases result in 200,000–400,000 deaths worldwide (Coutte and Locht 2015), of which 90% occur in developing countries, most of them in infants of very young age to have been vaccinated or to have a complete vaccination schedule (Beltrán-Silva et al. 2011).

Outbreaks and epidemics of pertussis occur in cycles every 3 to 5 years (Pérez-Pérez et al. 2015). Antigen variation may allow the development of disease in vaccinated individuals, but there is no direct evidence that it causes a vaccine failure (Kingston et al. 2014).

When pertussis is acquired by vaccination or through natural infection, immunity does not last forever. In order of appearance, immunity you get with natural infection goes from 3.5 to 30 years, but with acellular vaccine, from 4 to 7 years and with whole cell vaccine from 5 to 14 years; although acellular vaccines reduce side effects as well reduce the long-lasting response and cause of high costs due to the multiple administrated doses (Soria-Guerra et al. 2009).

The immunity that it's acquired with the vaccines and the natural infection, decreases over time, then adolescents and adults become susceptible to infection of *B. pertussis* (Kingston et al. 2014).

In a study focused on single dose vaccination of pertussis, Mielcarek et al. (2010) produced the attenuated live *B. pertussis* strain BPZE1 as a live vaccine candidate by genetically removing or altering three *B. pertussis* toxins: dermonecrotic toxin (DNT), pertussis toxin (PTX), and tracheal cytotoxin (TCT); this strain induced a dose-dependent protection after a single nasal dose challenge in mice.

Currently one of the challenges is the development of a low-cost pertussis vaccine which can be effective with a single dose, this is particularly important in the developing countries, since it is difficult to access health centers and to complete the vaccination scheme of infants. In the other hand, recently the use of plants as bioreactors has been explored as an alternative and less expensive platform capable of incorporating transgenes and expressing heterologous proteins that are immunogenic in mice (Soria-Guerra et al. 2007, 2011; Brodzik et al. 2009).

Plants provide many advantages over recombinant mammalian systems, including the absence of potential contamination with viruses and prions, a much higher scalable capacity, and very low costs of large-scale production (Kwon and Daniell, 2016; Ward et al. 2020; Rosales-Mendoza et al. 2021).

The plant transient transformation approach mediated by viral transfection, produced heterologous proteins in short time, higher levels of expression, inexpensive production, versatility and biosafety showing advantages over the other expression systems. An example of this is the magnICON system, developed by Icon Genetics, which has allowed the expression of the green fluorescent protein (GFP) in leaves of *Nicotiana benthamiana*, where observed up to 80% of total soluble protein (Marillonnet et al. 2004; Gleba et al. 2005). Additionally, the use of *Nicotiana tabacum* with a low alkaloid is recommended for production of recombinant proteins (Menassa et al. 2007).

In order to achieve our goal a transient transformation technology called magnICON was used for production of recombinant proteins of *B. pertussis* in the 81V9 cultivar *N. tabacum* plants as a bioreactor. This tobacco cultivar has shown to be one of the most effective candidates for production of recombinant proteins in tobacco due to its growth rate, high soluble protein levels, leaf biomass yield, and low alkaloid content and has been extensively used as a platform for transient and nuclear-transformed, stable expression systems (Gutiérrez et al. 2013; Kolotilin et al. 2013), and in addition, there is no need for subsequent purification of recombinant protein.

In the present study we describe the use of low-alkaloid *N. tabacum* plants through a transient expression system (the magnICON system) to express an optimized synthetic gene encoding a new immunogenic protein based in epitopes of pertactin, filamentous hemagglutinin and pertussis toxin proteins of *B. pertussis*, which were antigenic and immunogenic when were tested in mice. This proof of concept opens the possibility of the production of an edible subunit vaccine against pertussis.

Materials and methods

Gene design and vector construction

A chimeric protein was designed to include two epitopes of the S1 subunit of the pertussis toxin (Askelöf et al. 1990; Barbieri et al. 1992), the immunodominant protective type I domain (F1) of filamentous hemagglutinin (FHA) and the highly immunogenic region II domain (P2) of pertactin (PRN) (Zhao et al. 2009). The epitopes of the S1 subunit toxin and the fusion protein were joined by linkers rich in proline (GPGPG). These spacers enabled proper deployment of linear epitopes (Livingston et al. 2002). The coding gene was optimized for *Nicotiana tabacum* and synthesized by GenScrip Co. and named BPns (Fig. 1a). At the 3' end of the BPns gene, we added the SEKDEL sequences, that is an endoplasmic retention signal. At the 5' end a histidine tag and a site for protease were added, also we added a restriction sites for *Nco*I and *Sac*I enzymes for subsequent cloning (New England, Biolabs, USA).

To generate the pICHBPnsM3' module, the BPns gene was cloned into the vector pICH11599 (Fig. 1b) and it was used in the magnICON system (Marillonnet et al. 2004; Gleba et al. 2005). The positive clones were selected through PCR analysis, sequenced and the plasmids were then mobilized into *Agrobacterium tumefaciens* GV3101 cells via electroporation (Patiño-Rodríguez et al. 2013). Positive

GV3101 transformants bacteria were verified by restriction analysis. After that, positive clones were propagated at 28 °C in the Luria–Bertani (LB) medium containing rifampicin (100 mg/L) and kanamycin (100 mg/L) in order to perform the agroinfiltration how is described later on. All procedures were performed using standard techniques (Sambrook et al. 1989). In this work we also employed the following plasmids; pICH4851 as a 5' replicase module, pICH10881 as an integrase module and pICHGFP as a 3' GFP module (employed as a positive control). The in vivo recombination of the modules is aimed to produce the PTF-M3' protein in tobacco.

The same sequence of BPns gene was codon bias optimized for bacteria expression by GenScript (USA) to improve the gene translation of the PTF protein in *E. coli*, and the sequence was cloned in frame into the pET28b (+) vector (Novagen, <http://www.novagen.com>) through the *Xba*I and *Xho*I sites to obtain the rBPns expression vector. A positive clone was selected through a restriction analysis and used for bacterial recombinant protein (PTF) expression assays.

Production and purification of *E. coli*-derived PTF protein

E. coli TOP10F' strain (Invitrogen, <http://www.invitrogen.com>) was transformed with rBPns expression vector which produces the PTF protein, and cultured in LB medium with

Fig. 1 BPns gene and vector representation used for transient transformation. **a** BPns gene: His histidine tag, TEV protease site, PT1 and PT2 epitopes pertussis toxin S1 subunit, FHA/PRN fusion protein filamentous hemagglutinin and pertactin; L linker, SEKDEL endoplasmic reticulum retention signal. Restriction sites are indicated by arrows. **b** Constructs used in the current work: (1)–(2), 3' pro-vector modules: pICHBPnsM3' and pICHGFP; (3) 5' pro-vector module: pICH4851; (4) Integrase module: pICH10881. LB and RB binary vector left border and right border, respectively, Int intron, AttB and AttP DNA-specific recombination elements, NTR non-translated region, NOS nopaline synthase terminator, ACT2 Actin gene 2 promoter, RdRp RNA-dependent RNA polymerase, MP movement protein (Modified from Marillonnet et al. 2004)

100 mg/L of kanamycin. The PTF protein expression was induced with isopropyl-thiogalactoside (IPTG) 1 mM of final concentration. The purification of the PTF chimeric protein was performed by Ni-IMAC kit (QIAGEN, <http://www.qiagen.com>) according to Govea-Alonso et al. (2013). Briefly, bacterial cells were centrifuged to form a pellet, resuspended in cold TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8), and then lysed by sonication (Sonics Vibra-Cell Model VC130). Inclusion bodies were pelleted by centrifugation at 8000 rpm for 10 min, washed once with 0.5 M NaCl-1% Tween-20, and then twice with cold TE buffer. Inclusion bodies were solubilized in Urea buffer (100 mM NaH_2PO_4 , 7 M Urea, 0.5 M NaCl, 10 mM Imidazole, pH 8) and incubated with NiNTA resin by 1 h (QIAGEN, <http://www.qiagen.com>). The elution of the recombinant proteins was performed with binding buffer supplemented with 250 mM imidazole; in order to renature the recombinant proteins desalting and re-folding was required. The elution fractions were dialyzed in refolding buffer overnight (30 mM Na_2CO_3 , 70 mM NaHCO_3 , 7% sucrose, pH 9.6). Then the recombinant protein was clarified by centrifugation and stored at -80°C until used. Protein concentration was quantified by Bradford method (1976), then, purity of the protein was visualized by SDS-PAGE and confirmed by Western blot assays respectively (Fig. 3a and b).

Agroinfiltration

The Agroinfiltration was performed with 100 mL of an overnight grown culture in LB medium (Sigma-Aldrich, St. Louis, MO, USA) of *A. tumefaciens* GV3101, OD_{600} ranging from 1.6 to 2.5, then was centrifuged at 8000 rpm for 5 min to obtain the pellet, then was resuspended in 1 L of infiltration buffer, which contains 10 mM of 2-*N*-morpholinoethanesulfonic acid (MES) and 10 mM of MgSO_4 (Sigma-Aldrich, St. Louis, MO, USA) at pH 5.5. *N. tabacum* seeds cv. 81V9 with low-levels of nicotine, were donated by Dr. Menassa (Southern Crop Protection and Food Research Centre, ON, Canada). Ten seeds were germinated in 50% Murashige Skoog (MS) medium under aseptic conditions. The plants were grown in pots with soil for 4 weeks in an environmental chamber (16 h/8 h light/dark) at 25°C . The seedlings were gently removed from pots and roots washed in order to introduced the soil free leaves (upside down) into the vacuum chamber with the *Agrobacterium* solution, then agroinfiltrated in a solution of *Agrobacterium* cells (1.8×10^8 colony forming units per mL). Vacuum of 0.5–1 bar was applied for 1 min and then returned to atmospheric pressure and grown in an environmental chamber (16 h/8 h light/dark) at 25°C . After 7 days post infection (dpi), the infiltrated leaves were harvested and lyophilized for use in further studies. Six grams of lyophilized material was obtained for protein purification (Marillonnet et al. 2004).

Purification and quantification of tobacco-derived PTF-M3' protein

The lyophilized leaves were ground in liquid nitrogen and protein was extracted at 4°C with 30 mL PBS (100 mM NaCl, 10 mM Na_2HPO_4 , 3 mM KH_2PO_4 , pH 7.2) supplemented with 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA), and 30 μL Protease Inhibitor Cocktail (Sigma-Aldrich®), with constant stirring and then was centrifuged at 10,000 rpm for 10 min. The supernatant was loaded onto a column pre-loaded with NiNTA resin (Invitrogen, <http://www.invitrogen.com>) and washed with extraction buffer (50 mM NaH_2PO_4 , 0.5 M NaCl, 40 mM Imidazole, pH to 7.5) and then the recombinant protein was eluted with this buffer supplemented with three different concentrations of Imidazole (50 mM NaH_2PO_4 , 0.5 M NaCl, pH to 7.5, and 100-, 300- and 500-mM Imidazole) and fractions of the purified recombinant protein were collected.

PTF-M3' protein concentration was estimated by indirect-ELISA. Standard curve was made in a 96-well plate with serial dilutions of 500 to 1 ng/mL of a control protein with a histidine-tag (SdrP from *Thermus thermophilus*). Samples were diluted in carbonate buffer and incubated 12 h at 4°C . Washes were performed with TBS 0.1% Tween 20. Blocking was performed with buffer A (15 mM $\text{NaB}_4\text{O}_7 + 120$ mM de NaCl + 0.25% BSA + 0.05% Tween 20 + 1 mM EDTA + 0.05% NaN_3 , pH: 8.5). Then, plaque was incubated with an Anti-His Monoclonal Antibody from mouse (A00186-100, GeneScrip Inc, Piscataway, NJ, USA) at 1:3000 dilution for 2 h. After, wells were incubated an Anti-Mouse Alkaline Phosphatase antibody produced in rabbits (A1902; Sigma Aldrich, St Louis, MO, USA) at 1:1500 dilution. Colorimetric reaction with 3 mM solution of p-nitrophenyl phosphate was read at 405 nm in iMarTM Microplate Absorbance Reader (Bio-Rad, Hercules, CA, USA). The concentration of the peptide in the extracts was determined by linear regression. The data were analyzed for paired *t* test analysis ($P < 0.05$ was considered as a statistically significant difference). The statistical analysis and graphic were made in GraphPad Prism 6 Software.

PTF-M3' purity was determined by SDS-PAGE and confirmed by Western blot assays respectively.

Detection of chimeric proteins

The purified proteins obtained from infiltrated leaves and from *E. coli*, were detected by Western blot analysis. Twenty microliters of each purified protein were resuspended into 20 μL of Laemmli buffer (Sambrook et al. 1989), then samples were boiled at 95°C for 10 min. The proteins were resolved into a 12% polyacrylamide gel (SDS-PAGE) under denaturing conditions. The blotting was performed onto a BioTrace polyvinylidene fluoride (PVDF) membrane (Pall

Corporation, Cortland, NY), by a Mini-Trans-Blot Electrode Module (BioRad) with a transfer buffer (20 mM Tris, 150 mM glycine and 20% methanol). After blocking non-specific sites with PBS plus 3% nonfat milk, blot was incubated overnight at 4 °C with a monoclonal mouse anti-His tag antibody (1:5000 dilution; Serotec). A horseradish peroxidase conjugated anti-mouse antibody (1:10,000 dilution; A5420; Sigma-Aldrich) was incubated during 2 h at room temperature. Specific antibody detection was completed with the SuperSignal West Dura kit (Thermo Scientific, <http://www.thermoscientific.com>).

Immunization of mice

All animal experiments were performed on accordance with Mexican federal regulations for animal experimentation and care (NOM-062-ZOO-1999, Ministry of Agriculture, Mexico). Male Balb/c mice between 8–9 weeks old, were used and randomly divided into four different groups ($n=5$) and received the following treatments: 5 µg of *E. coli*-derived of rBPns (group PTF), 5 µg of purified protein of infiltrated leaves (group PTF-M3'), the purified proteins were co-administrated with 10 µg of Cry1Ac protoxin from *Bacillus thuringiensis* as an adjuvant (Moreno-Fierros et al. 2003), 5 µg of Boostrix® vaccine (group BSX) (GlaxoSmithKline Biologicals SA, Belgium), and the mock group (vehicle alone, group PBS). Four doses were administered by gavaged in liquid form to each group on days 0, 7, 14, and 21. At day 28, a boost was administrated intranasal with the same doses of all antigens in order to expect a respiratory mucosal response (Birkhoff et al. 2009; Lobiana-Mato 2019). At the end, on day 32, the mice were euthanized to collect serum samples, gut and tracheopulmonary fluids.

Serum and intestinal fluid collection and preparation

Following cardiac puncture from ether-anesthetized mice, serum samples were obtained from blood extracted. Tracheopulmonary fluid was collected by surgical technique provided by Dr. Andreu Garcia-Comas from Center for Research on Infectious Diseases of National Institute of Public Health, Cuernavaca, Mexico. Briefly, the trachea and lungs were exposed, a cannula was inserted into the trachea and 1 mL of cold PBS supplemented with 1 µL of Protease Inhibitor Cocktail (Sigma-Aldrich Chemical Co., St Louis, MO) was flushed through the trachea to the lungs and this was re-extracted and 500 µL were collected for each mouse. According to Moreno-Fierros et al. (2000), fluids from the gut were collected. The small intestines were flushed out with 3 mL of cold PBS supplemented with 1 µL of Protease Inhibitor Cocktail (Sigma-Aldrich Chemical Co., St Louis, MO). Then the washes were centrifuged at 4 °C for 10 min

at 12,000 rpm and supernatants were frozen immediately and stored at – 70 °C until used for analysis.

Immunoenzyme assays

An enzyme-linked immunosorbent assay (ELISA) was performed in order to quantify the antibody levels in serum, intestinal and lung fluids. Briefly 96-well plates were coated with 1 µg of PTF protein in 100 µL per well in bicarbonate-carbonate buffer (15 mM Na₂CO₃, 35 mM, NaHCO₃, pH 9.6). Plates were incubated overnight at 4 °C and then washed three times with PBST (0.05% v/v Tween-20 in PBS). We used 5% nonfat dry milk in PBS for blocking non-specific binding. The serum was diluted 1:10 in PBS, whereas gut and tracheopulmonary fluids were diluted 1:2 in PBS and added to microwells. Duplicates of each sample were incubated overnight at 4 °C and washed three times with PBST. Rabbit anti-mouse IgG alkaline phosphatase antibody or anti-IgA (Sigma-Aldrich Chemical Co., St Louis, MO) or goat polyclonal secondary antibody to mouse IgG1 and goat polyclonal secondary antibody to mouse IgG2a (Abcam, Cambridge, MA) were used as secondary antibodies. The plates were incubated for 1 h at 37 °C, and then were washed and the enzymatic reaction was developed with substrate solution following the manufacturer's instructions (Agdia, <http://www.agdia.com>). The OD at 450 nm was recorded in a microplate reader (Microplate Reader 550, Bio-Rad Laboratories, Inc.).

Statistical analysis

For each group, data were expressed as the mean value $\pm$ SD. Statistical differences among groups were evaluated with a one way-ANOVA analysis ($P < 0.001$), with Tukey multiple comparison test (95% confidence). Differences were considered statistically significant for different letters. Treatment with the highest difference is showed with letter a. GraphPad Prism (Version 9.1.0 -216) and Minitab Express (Version 1.5.1) packages were used for statistical analyses.

Results

Design and cloning of the *Bordetella pertussis* synthetic gene for nuclear expression (BPns)

We selected epitopes from these three virulence targets: PT which is the most complex virulence factor expressed by *B. pertussis* previously used in an acellular pertussis vaccine formulation (Stein et al. 1994). FHA which is the major *B. pertussis* adhesion protein. Mature PRN, is a 69-kDa protein expressed on bacterial surface, which has been proposed to play a role in attachment (Fedele et al. 2013). Therefore,

we designed a multiepitope polypeptide, as a subunit vaccine candidate for whooping cough. To construct a putative highly antigenic chimeric protein, we selected the first 17 amino acids and 17 additional amino acids (positions 99–115) of the pertussis toxin, 465 base pair fragment that specified the important immunodominant type I domain of the FHA and the 300-base pair fragment of the immunodominant region II of the pertactin. In addition, we included proline-rich linkers and at the C-terminal an endoplasmic retention signal SEKDEL. In the BPns gene six histidine tag was placed at the 5' end, and downstream the His-tag sequence, a site for TEV protease was placed. Codon optimization for better plant expression was achieved without changing the amino acid sequences of the native exotoxins and was conducted by the company GenScript. Restriction sites were introduced at the 5' and 3' ends for *Nco*I and *Sac*I, respectively (Fig. 1a), for convenient cloning into magnI-CON expression vectors.

PTF production in *E. coli*

In order to compare the biological activity of the recombinant protein produced in plants, a multicomponent fusion protein produced in *E. coli* (PTF) (codon bias for bacteria expression) was designed as a vaccine candidate against pertussis. The bacterial optimized BPns gene was successfully assembled and cloned into a bacterial expression vector and

transferred into *E. coli*. PTF protein was successfully over-expressed through IPTG induction assay. The protein PTF was purified by immobilized metal affinity chromatography (IMAC) and detected by SDS-PAGE (Fig. 2a) and Western blot analyses (Fig. 2b) using a monoclonal mouse antibody directed against the Histidine tag (1:5000 dilution; Serotec). A single band of the expected size 33 kDa was detected (Fig. 2b).

BPns gene expression in *N. tabacum*

The vectors employed in this study are shown in Fig. 1b. We show two constructs pICHBPnsM3' (Fig. 1b1) and pICH-GFP (Fig. 1b2) with the gene of interest and the reporter gene respectively, and both were transfected in combination with the vector modules pICH4851 (Fig. 1b3) and pICH10881 (Fig. 1b4) in leaves of *N. tabacum* plants grown under greenhouse conditions. At 7 days' post-infection (dpi) the plant disease symptoms were observed, such as leaf yellowing and curling. Plants were infected with the vector contained GFP as control. The expression of GFP was observed under UV light in plants infected with this control (Supplementary Fig. 1), suggesting the proper virus replication and the expression of this transgene in *N. tabacum*.

Fig. 2 Production and detection of the purified PTF protein in *E. coli*. **a** PTF purified from *E. coli* was visualized by SDS page stained by Coomassie Blue. Lanes: M, BenchMark™ Pre-stained protein ladder; 1 negative control (total soluble protein from untransformed *E. coli*); 2 protein PTF purified. **b** Presence of the expected recombinant PTF

was determined by Western blot analysis using a monoclonal mouse antibody directed against the Histidine tag. Lanes: 1 negative control (total soluble protein from untransformed *E. coli*); 2 protein PTF purified

PTF-M3' purification

After 7 dpi, the tobacco leaves were harvested and purified by IMAC assay. The presence of recombinant PTF-M3' plant protein of 30 kDa was confirmed by an SDS-PAGE (Fig. 3a) and a Western blot analysis with an anti-His monoclonal antibody (Fig. 3b) and using as a positive control the bacterial PTF protein. Additionally, an ELISA was performed to estimate the recombinant protein concentration and yield (Fig. 4), rendering 0.443 mg of PTF-M3' per mL and a yield of 0.0739 mg of protein per g fresh weight. This is a similar amount of recombinant protein produced in an improved protocol to obtain very high levels in *E. coli* (Sivashanmugam et al. 2009) and also similar to those produced by Regnard et al (2010) using an autonomously replicating geminivirus-derived shuttle vector, therefore our transformation strategy can be a promising approach to produce good levels of recombinant proteins.

Serum antibody response

To evaluate immunogenic responses of the bacterial PTF and plant PTF-M3' proteins, we proceeded to immunize mice

Fig. 4 Quantification of PTF-M3' plant protein by ELISA. The PTF-M3' protein eluted in 500 mM imidazole and TSP from wild type plant were assayed by an anti-histidine antibody. PTF-M3'1, PTF-M3' plant protein; WT, Wild type plant extract. The results show the means $\pm$ standard deviations (SD) of three independent assays. The results were analyzed by paired *t* test, statistical significantly is ($P < 0.05$) (***) (***0.001)

Fig. 3 SDS-PAGE and Western blot assays of purified protein agroinfiltrated plants 7 dpi. **a** Protein samples were subject to sodium dodecylsulphate 12% polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, stained with Coomassie Blue and blotted on to a nitrocellulose membrane. 1 Elution with 500 M imidazole of

the BPnsM3' construct; 2 Wild type plant extract.; M, Benchmark™ Pre-stained protein ladder. **b** The membrane was probed with a monoclonal mouse antibody 1 directed against the Histidine tag. M, molecular weight standard; 1 protein PTF purified; 2 Elution with 500 M imidazole of the BPnsM3' construct; 3 Wild type plant extract

with these proteins. In this study, BALB/c mice were immunized with four oral doses and one intranasal dose of either the vehicle alone (PBS) or with the purified proteins, all co-administered with 10 μ g of Cry1Ac protoxin from *Bacillus thuringiensis* as mucosal adjuvant. As is shown in Fig. 5, mice immunized with the PTF-M3', the commercial vaccine Boostrix®, and the bacterial PTF protein, elicited serum IgG responses, showing a statistically significant difference ($P < 0.05$) in comparison to the vehicle alone. It is interesting to highlight, that the highest response was observed with the PTF-M3' protein.

IgG antibody subclasses in serum, tracheopulmonary and intestinal fluid

Specific serum antibodies IgG1 (Fig. 6 grey bars) and IgG2a (Fig. 6 dark bars), were elicited in mice immunized with all antigens, showing a statistically significant difference in comparison to the vehicle alone ($P < 0.05$). Overall, an apparent balance between the two antibody responses was detected suggesting a balanced Th1/Th2 response. Altogether, the above-mentioned results indicated that oral delivery of PTF and PTF-M3' was immunogenic, and the highest response was observed after immunization with the recombinant protein produced in plants.

Fig. 5 Serum specific antibody responses, against PTF protein. Four-Weekly doses of the vehicle alone (PBS), Boostrix vaccine (5 μ g, BSX), purified protein of infiltrated leaves (5 μ g, PTF-M3'), and the purified bacterial protein (5 μ g, PTF) were administered to male BALB/c mice via the oral route and one intranasal booster. The PTF and the PTF-M3' groups were co-administered with 10 μ g of Cry1Ac protoxin from *Bacillus thuringiensis* as an adjuvant. IgG antibody levels were determined by ELISA in serum samples diluted 1:10. Individual samples were run in duplicate. Mean A405 values $\pm$ SD, from each experimental group ($n=5$) are shown. Statistical differences among groups were evaluated with a one way-ANOVA analysis ($P < 0.001$), with Tukey multiple comparison test (95% confidence). Differences were considered statistically significant for different letters. Treatment with the highest difference is showed with letter a

Fig. 6 IgG subclasses of antibodies from serum. Four weekly doses of Boostrix vaccine (5 μ g, BSX), purified protein of infiltrated leaves (5 μ g, PTF-M3'), purified bacterial protein (5 μ g, PTF) and the vehicle alone (PBS) were administered to male BALB/c mice by the oral route and one intranasal booster. IgG subclasses antibody levels were determined by ELISA in serum samples diluted 1:10. Mean A405 values $\pm$ SD, from each experimental group ($n=5$) are shown. **a** IgG1 subclass and **b** IgG2a subclass. Statistical differences among groups were evaluated with a one way-ANOVA analysis ($P < 0.001$), with Tukey multiple comparison test (95% confidence). Differences were considered statistically significant for different letters. Treatment with the highest difference is showed with letter a

The IgG levels in the tracheopulmonary fluids (TPF) were low in all groups test in comparison to the serum ones ($P < 0.05$; Fig. 7a), nevertheless, PTF-M3' showed the highest response. On the other hand, IgA responses in TPF from mice immunized with PTF, PTF-M3' and the vaccine Boostrix® by oral route were better than for IgG (Fig. 7b), but moderate in comparison to the serum responses. The antibody responses found and the intestinal fluids (Fig. 8) were higher than those in TPF. In this treatment the PTF-M3' protein elicited the highest response only in the IgA antibody level in comparison to the rest of the treatments (Fig. 8b).

Discussion

The triple vaccine for diphtheria, pertussis and tetanus (DPT) has been extensively employed in infants and children around the world. Although its efficacy is well documented side effects continue to be reported and efforts have been made to produce acellular vaccines that reduce these undesirable effects; however, currently DPT acellular vaccine production is an expensive process, which involves the utilization of highly cost reactor systems and purification processes of recombinant proteins of three different bacteria.

Thanks to the improvement of molecular biology techniques, new strategies for the production of subunit vaccines in plants have been developed and have been suggested as

Fig. 7 Antibody responses in lungs. Four weekly doses of Boostrix vaccine (5 µg, BSX), purified protein of infiltrated leaves (5 µg, PTF-M3'), purified bacterial protein (5 µg, PTF) and the vehicle alone (PBS) were administered to male BALB/c mice via the oral route and one intranasal booster. **a** IgG and **b** IgA antibody levels were determined by ELISA in tracheopulmonary fluids diluted 1:2. Individual

samples were run in duplicate. Mean A492 values $\pm$ SD, from each experimental group ($n=5$) are shown. Statistical differences among groups were evaluated with a one way-ANOVA analysis ($P<0.001$), with Tukey multiple comparison test (95% confidence). Differences were considered statistically significant for different letters. Treatment with the highest difference is showed with letter a

Fig. 8 Antibody responses in intestinal fluids. Four weekly doses of Boostrix vaccine (5 µg, BSX), purified protein of infiltrated leaves (5 µg, PTF-M3'), purified bacterial protein (5 µg, PTF) and the vehicle alone (PBS) were administered to male BALB/c mice by the oral route and one intranasal booster. **a** IgG and **b** IgA antibody levels were determined by ELISA in intestinal washes diluted 1:2.

Individual samples were run in duplicate. Mean A492 values $\pm$ SD, from each experimental group ($n=5$) are shown. Statistical differences among groups were evaluated with a one way-ANOVA analysis ($P<0.001$), with Tukey multiple comparison test (95% confidence). Differences were considered statistically significant for different letters. Treatment with the highest difference is showed with letter a

an efficient platform for the production of antigens because they have been found to be safe and induce sufficiently high immune response (Tiwari et al. 2009).

Furthermore, at the laboratory level there are reports in which it is shown that expressing synthetic genes that encode proteins of bacterial or viral origin that were engineered for expression in plants, greatly improves the expression of the heterologous protein (Haq et al. 1995) and the expression of relevant epitopes rather than whole protein, reduces the risk of causing adverse reactions at low cost (Yu and Landrige 2001; Guan et al. 2013).

Soria-Guerra et al. (2007) produced transgenic tomato plants expressing an optimized synthetic gene encoding a polypeptide with epitopes of the DPT exotoxins under the control of CaMV35S promoter. After this report, the expression of DPT recombinant antigenic components in low-alkaloid tobacco and carrot cell cultures was described as an alternative to production of a recombinant DPT subunit vaccine (Brodzik et al. 2009).

Additionally, Soria-Guerra et al. (2011), demonstrated that oral immunization of Balb/c mice with transgenic tomatoes harboring the DPT polypeptide elicited specific

and mucosal antibody responses, however pertussis level antibodies produced were low.

Because of pertussis is considered a reemerging disease, in this work we propose the possibility of generating a subunit vaccine in plants with relevant epitopes of *B. pertussis* with putative immunoprotective properties, expressing the main agents of pathogenicity, as a proof of concept, which is the initial phase for the development of an oral vaccine.

Our results showed humoral responses elicited in mice, both at mucosal and systemic levels, and we demonstrated that PTF-M3' produced in tobacco leaves with magnICON system is immunogenic, with higher responses than the commercial vaccine Boostrix®, in addition, the serum antibody response levels are similar to previous reports (Rosales-Mendoza et al. 2008) where a protection effect was demonstrated, therefore the PTF-M3' recombinant protein produced in this work, could be used for eliciting neutralizing antibodies in future studies.

The immunoadjuvant properties of Cry1Ac protoxin from *Bacillus thuringiensis* have previously proven (Vazquez et al. 1999), therefore our recombinant proteins were co-administrated with the protoxin Cry1Ac (Moreno-Fierros et al. 2003).

We used an immunization schedule of four oral doses and a fifth booster immunization was administered by the intranasal route. This was done based on the low IgG levels detected after the four oral doses (data not shown).

Results suggested that the recombinant PTF-M3' and PTF proteins induced specific systemic humoral immune responses in vaccinated mice. To determine if our vaccine can provide an effective protective immunity, it will be necessary to challenge Balb/c animals with a lethal dose of *B. pertussis*.

To our knowledge, this is the first report of the expression of a recombinant fusion protein harboring three relevant *B. pertussis* epitopes: the S1 subunit of the pertussis toxin, the filamentous hemagglutinin (FHA), and the highly immunogenic region II domain (P2) of pertactin (PRN) that could function as a subunit vaccine against whooping cough, expressed in plants by transient transformation, whose oral immunogenicity with the infiltrated leaves on mice was demonstrated. In a future work it would be important to test if PTF-M3' protein has immunoprotective effects by a challenge assay.

Currently companies such as MedicaGo are investing in vaccine research using plants as recombinant protein platform production and they have published a work on influenza vaccine produced in *Nicotiana benthamiana* stating that it is “the largest demonstration to date of the potential for a plant-based platform to produce a human vaccine that can be safe, immunogenic, and effective” (Ward et al. 2020), in addition they are in clinical trials for a COVID-19 vaccine, because the production costs are lower than other platforms.

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Author contributions KSA, LMF, RESG and AGAS conceived and designed research. KSA, SRM and KLRB conducted experiments. EME and RCC contributed new analytical tools. KSA, AGAS analyzed data. KSA and AGAS wrote the manuscript. All the co-authors contributed substantially to revisions and approved the manuscript.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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