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Production and immunogenicity assessment of a ToxA-based multiepitope plant-made protein targeting enteric pathogens

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Abstract

Around 1.7 million people die annually due to enteric infections, which are mainly caused by ETEC (Enterotoxigenic *E. coli*), *V. cholera*, *V. parahaemolyticus*, *and Salmonella*. There are currently licensed vaccines against cholera and salmonella, whose distribution is chain-dependent making difficult to efficiently distribute them in poor countries. In this context plants are attractive hosts for the synthesis and delivery of subunit vaccines that could be produced at very low costs and widely distributed ensuring vaccination coverage. Subunit vaccines often demand the use of adjuvants to reach proper immunogenicity. Several bacterial toxins have been used as mucosal and systemic adjuvants and a potential molecule for this purpose is the PirA-like toxin (ToxA) from *V. parahaemolyticus*, which was deemed highly immunogenic in some species. In this study a protein named *ToxAentero* was expressed in tobacco plants to initiate the development of accessible vaccines against enteric diseases. *ToxAentero* is based on ToxA, as adjuvant carrier, and epitopes from ETEC, *V. cholerae*, *V. parahaemolyticus*, and *S. typhimurium*. The production yields reached up to 5.46 μg g^{-1} fresh leaf tissue. The plant-made *ToxAentero* was found immunogenic in mice immunized by oral or subcutaneous routes in terms of the induction of IgG (sera) and IgA (feces) humoral responses against most of the target epitopes from the enteric pathogens. This study opens the path for the development of a promising oral plant-based multiepitopic vaccine candidate in the fight against enteric diseases.

Key message

A multiepitopic protein targeting several enteric pathogens, was expressed in plants and characterized in mice; revealing that it is immunogenic and thus is proposed as an attractive vaccine candidate.

Keywords ToxA · adjuvant/carrier · Plant-based vaccine · Multiepitopic · Enteric diseases

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Introduction

Diarrhea is still a major sanitation disease problem associated to around 1.7 million of deceases each year. There are several pathogens causing diarrhea; however, ETEC and *Vibrio cholerae* are the commonest bacteria-causing diarrhea. These bacteria are responsible for around 500,000 deaths of children annually (WHO 2017). Other important enteric pathogens are: Salmonella that causes 1.4 million infections each year in the United States (The Center for Disease Control and Prevention, 2020) and *Vibrio parahaemolyticus* that is associated with 30% of the food-related poisonings in Japan (Broberg et al. 2011). Both pathogens persist as major public health threats in developed and low-income countries.

There are two vaccines used to fight against cholera: (1) Shanchol® killed bivalent (O1 & O139) whole cell oral cholera vaccine and (2) Dukoral® composed of inactivated *V. cholerae* serotype O1 and recombinant CTB (Bi et al. 2017). Currently, an ETEC vaccine is not available in the market, nonetheless advanced clinical trials have been performed. Meanwhile, Dukoral® has been used against ETEC with a 75% protection rate. There are two vaccines licensed for use against *Salmonella*. These are the live attenuated vaccines: Ty21a and Vi capsular polysaccharide (Vi CPS), which only protect about 50% immunized people; having poor immunogenicity in young children (Benoun et al. 2018). However, both vaccines target *S. typhi* infection and no vaccines are available against *S. paratyphi, S. typhimurium*, and *S. enteritidis* (MacLennan et al. 2014). Similarly, vaccines to prevent *V. parahaemolyticus* infection have not been licensed and the field is limited to some candidates evaluated at the preclinical level (Li et al. 2014). Overall, these vaccines require cold chain for storage and distribution; limiting vaccine coverage in low- and middle-income countries (Ashok et al. 2017).

In this arena, plants are well-proven hosts for the production and oral delivery immunogens; several plant-made proteins have been assessed as vaccine candidates against enteric and respiratory diseases (Azegami et al. 2020). The use of plant-made immunologically active antigens offers several advantages that include low production costs, cold chain-free formulations, easy scalability, no replication of human pathogens, and proper synthesis of complex heterologous proteins (Rosales-Mendoza et al. 2016; Criscuolo et al. 2019; Kurup and Thomas 2020). Today; molecular biology, genetic engineering, and epitope mapping allow for the effective design and recombinant expression of multiepitopic recombinant antigens capable of triggering immunity against several pathogens using a single antigen (Ruan et al. 2015).

Vaccine subunits usually require immunostimulant elements to potentiate the immune response, especially in oral immunization schemes (Chauhan et al. 2017). In this regard, several bacterial toxins have been used as mucosal adjuvants; including the B subunits of the cholera toxin (CTB) or the heat labile enterotoxin (LTB) (Kang et al. 2004; Adkins et al. 2012; Al-Barwani et al. 2014). Both subunits increase secretory antigen-specific IgA responses and cellular immunity (Kang et al. 2004). Recently another subunit toxin, the PirA-like toxin (ToxA) from *Vibrio parahaemolyticus*; has been reported to elicit immunostimulatory and protective effects in shrimp and fish against bacterial challenges (Campa-Córdoba et al. 2017; Reyes-Becerril et al. 2016; 2017). In fish (*Lutjanus peru* and *Sparus aurata*), an intraperitoneal injection of ToxA induced mucosal and systemic humoral responses (IgM) with the expression of proinflammatory cytokines (IL-1β, TNF-α) (Reyes-Becerril et al. 2016, 2017). ToxA is safe upon oral or intraperitoneal administration in shrimp and fish, respectively. Interestingly, ToxA has almost an identical conformational structure to the Cry toxin of *Bacillus thuriengiensis* (Lee et al. 2015), which is recognized as a potent systemic and mucosal adjuvant assessed in several vaccine candidate formulations (Moreno-Fierros et al. 2003, 2015; Rubio-Infante and Moreno-Fierros 2016). In line with these findings, Monreal-Escalante et al. (2019) demonstrated that a plant-made ToxA is immunogenic in orally immunized mice through the induction of systemic and mucosal antibody responses. The immunogenic properties of ToxA and its functional production in plant cells for oral delivery have opened an attractive path for several applications in human health. Therefore, the objective of this study was developing a plant-based multiepitopic protein (*ToxAentero*) based on antigenic determinants from enteric pathogens and evaluate its immunogenicity in mouse. *ToxAentero* comprised PirA-like toxin as adjuvant/carrier and epitopes from ETEC, *V. cholerae*, *V. parahaemolyticus*, and *S. typhimurium*. The expression levels of the recombinant *ToxAentero* produced in tobacco plants were determined and its immunogenicity assessed in terms of the induction of humoral responses in mice.

Materials and methods

Multiepitopic *ToxAentero* gene and molecular cloning

The multiepitopic gene was *in silico* designed based on a scrutiny of previously reported antigens and epitopes. The resulting gene coding for the target multiepitopic comprises epitopes located at ST (epitope sequence:

SNSSNYCCELCCNPACTGCYV) from ETEC, CT (epitope sequence: VEVPGSQHIDSQKCT) from *V. cholerae*, LptD (epitope sequence: WENQAIGSTGSSPEY) from *V. parahaemolyticus*, and FliC (epitope sequence: VQNRF-NSAITNLGNT) from *S. typhimurium* (Jacob et al. 1983, 1985; Newton et al. 1989; Bergman et al. 2005; Kremer et al. 2011; Rosales-Mendoza et al. 2011; Zha et al. 2016) (Table 1). The coding sequence of PirA-like toxin (ToxA) was used as immunogenic carrier; followed by a proline-containing linker (GPGP) to favor appropriate folding and displaying of the target epitopes. Finally, the coding sequence of the signal peptide from the *Glycine max* vegetative storage protein was incorporated at the N-terminus of the chimeric protein, whereas the sequence coding the SEK-DEL endoplasmic reticulum retention signal was included at the C-terminus. The gene was flanked by the *Sac*I and *Sma*I restriction sites to allow direct cloning into the pBI121 binary vector, driven by the CaMV 35 S promoter (Fig. 1). The *ToxAentero* coding gene (639 bp) was codon-optimized according to the expression host and synthetized by Gen-Script® (Piscataway, NJ.). Subcloning was carried out by conventional molecular cloning techniques to obtain the plasmid called pBI121*ToxAentero* (Sambrook and Russell 2006).

Agrobacterium-mediated tobacco transformation

The recombinant plasmid obtained from *E. coli* Top 10 cultures was identified by *Cla*I restriction analysis and subsequently mobilized into *Agrobacterium tumefaciens* (GV3101 strain) by electroporation. A positive *A. tumefaciens* clone was propagated and used for the tobacco transformation procedure.

Transgenic tobacco lines (*Nicotiana tabacum* cv. Petite Havana SR1) were transformed according to the method of Horsch et al. (1985). Briefly, tobacco leaf sections from in vitro-generated plants were infected with an overnight-grown culture of recombinant *Agrobacterium* ($OD_{600nm} = 0.5$) and co-cultured onto RMOP medium MS medium containing 3% sucrose, 1 mg/L benzyladenine, 0.1 mg/L naphthaleneacetic acid, 1 mg/L thiamine, and solidified with 3% agar; pH 5.8) at 25 °C for 48 h. Thereafter, selection and shoot development were accomplished by a selective medium (RMOP medium plus 250 mg/L cefotaxime and 100 mg/L kanamycin). Shoots were rooted in MS medium and regenerated plants were moved to soil and grown under a 16 h photoperiod, light intensity of 100 μmol $m^{-2} s^{-1}$, and 30% of relative humidity).

ToxAentero transgene detection

Total DNA was isolated from the candidate lines or wild-type (WT) plants by the method of Dellaporta et al. (1983). Detection of the transgene was performed by PCR analysis using the following primers: forward 5'CGCACAATCCCAC-TATCCTTCGC 3', targeting the 35 S promoter, and reverse 5'AGGGTTTCGCTCATGTGTTGAGC 3'; targeting the terminator NOS. Twenty five μL PCR reactions were set with 100 ng of test DNA, 1× PCR buffer, 1.5 mM magnesium chloride, 2.5 U Taq DNA polymerase (Vivantis), 1 mM dNTPs, and 1 μM of each sense and antisense primers designed to yield 769 bp amplicon. Temperature cycling conditions were: 94 °C for 5 min (initial denaturation), 35 cycles comprising 30 s at 94 °C (denaturation), 60 s at 56 °C (annealing), and 60 s at 72 °C (elongation); and a final extension at 72 °C for 5 min. Thermal cycling was performed in a MultiGene™ Mini Personal Thermal Cycler (Labnet). The presence of *ToxAentero* amplicons was assessed by electrophoresis using 1% agarose gels.

Detection of plant-expressed *ToxAentero*

In order to detect and quantify the recombinant *ToxAentero* in tobacco plants, a hyperimmune serum was generated in a mouse using pure recombinant ToxA; following a previously reported protocol (Ríos-Huerta et al. 2017). Recombinant ToxA was obtained as Monreal-Escalante et al. (2019) previously reported. Briefly, The *E. coli* BL21 strain carrying the pET-28a_ToxA expression vector, was used to produce recombinant ToxA. The *E. coli* BL21 strain was grown in Luria-Bertani (LB) medium supplemented with 50 mg/L ampicillin, at 37 °C, until it reached an OD600nm = 0.5. Expression was induced with 1 mM IPTG for 24 h at 37 °C. Cells were subsequently lysed by sonication at a 30% amplitude for eight 32 s-periods using an GEX130PB ULTRA-SONIC Processor. After clarification, the supernatant was collected and used for purification of the recombinant ToxA protein by Immobilized-Metal Affinity Chromatography (IMAC), using a BioRad NGC Quest equipment.

Table 1 Epitopes from four different enteric pathogens selected for the multiepitopic test vaccine

Patogen	Antigen	Epitope	Reference
ETEC/*V. cholerae*	LT/CT	VEVPGSQHIDSQK	Jacob et al. 1983, 1985; Newton et al. 1989
ETEC	ST	SNSSNYCCELCCNPACTGCYV	Rosales et al. 2011
V. parahaemolyticus	LptD	WENQAIGSTGSSPEY	Zha et al. 2016
S. Typhimurium	FliC	VQNRFNSAITNLGNT	Bergman et al. 2005; Kremer et al. 2011

(a)

(b)

Fig. 1 a Description of the expression vector used to produce the target antigen in tobacco plants. The *ToxAentero* gene was inserted into the pBI121 vector in which the expression is driven by the constitutive 35SCaMV promoter. The vector possesses *nptII* as selectable marker for kanamycin resistance. The multiepitopic gene *ToxAentero* contains the signal peptide from the *Glycine max* vegetative storage protein, the full-length sequence of the *V. parahaemolyticus* PirA-like toxin(ToxA), and a four-amino acid linker; followed by the target epitopes from LT/CT, ST, LptD, and FliC. The SEKDEL endoplasmic reticulum retention signal was linked at the C-terminus. **b** Detection of the *ToxAentero* gene in transformed plants. PCR analysis was performed for total DNA samples from candidate lines (P1-P11), WT (C-) plant and a purified pBI121-*ToxAentero* vector (C+) using specific primers targeting the 35 S promoter and the NOS terminator to detect the transgene, which results in a 769 bp amplicon

(a)

Animal handling was in accordance with the Guide for Care and Use of Laboratory Animals of the National Institute of Health (USA) and experiments accepted by the Committee on Research Ethics of the Faculty of Chemistry/University of San Luis Potosi (Permit Number: CEID-2013-004). The recombinant protein was detected in the transformed plants by Dot and Western blot analyses. Leaf tissues from transgenic or WT plants (100 mg) were pulverized in 300 µL of cold protein extraction buffer (750 mM Tris-HCl, pH = 8.0, 15% sucrose, and 1 mM PMSF. For Dot blot analysis extracted proteins were placed onto a BioTrace PVDF membrane (Pall Corporation, NY) and pure recombinant ToxA was used as positive control. After blocking (incubation in a fat-free dry milk solution, 5%, at 25 °C for

5 h), the membrane was incubated at 4 °C for 12 h with the anti-ToxA serum (1:500). For Western blot analysis, protein samples were mixed with 1× reducing buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol). The samples were boiled at 95 °C for 5 min and subsequently subjected to denaturing SDS-PAGE (4–12% acrylamide gels). The gel was transferred onto a BioTrace PVDF membrane (Pall Corporation, NY) using a TV100-EBK Electroblotter (AlphaMetrix Biotech, GER) in a methanol-based transfer buffer at 150 V for 1 h. After blocking (incubation in 5% fat-free dry milk at 25 °C for 5 h), the membrane was incubated overnight at 4 °C with the anti-ToxA serum (1:500). Pure recombinant ToxA was used as positive control. Immunoreactivity in both analyses was revealed by labeling with a horseradish peroxidase-conjugated goat anti-mouse antibody (1:2000; Sigma, St. Louis MO, USA) upon incubation for 2 h at room temperature; with a subsequent incubation with the SuperSignal West Dura solution according to the instructions from the supplier (Thermo Scientific, Waltham MA, USA).

ELISA was conducted to quantify *ToxAentero* levels in plant leaf tissues. Fifty milligrams of leaf tissue from transgenic or wild-type plants were pulverized with 500 µL of cold protein extraction buffer. Extracts were clarified by centrifugation (16,000×g at 4 °C for 15 min) and the supernatants diluted (1:16) in phosphate buffer saline (PBS) to be adsorbed on ELISA plates (4 °C, overnight incubation). Three washes with PBS + 0.1% Tween (PBST) were conducted after completing each step. The plates were blocked at room temperature for 2 h with a PBS solution containing fat-free dry milk (5%). Thereafter, anti-ToxA serum (diluted 1:500) was added and plates incubated at 4 °C overnight. Secondary labeling was performed with a goat anti-mouse horseradish peroxidase-conjugated antibody diluted 1:2000 (Sigma, St. Louis, MO; incubation at 25 °C for 2 h). The final step comprised a 30 min-incubation with an ABTS solution (0.3 mg/mL 2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Sigma, St. Louis, MO) and 0.1 mM H_2O_2). Optical density (OD) readings were obtained at 405 nm in a Multiskan® FC microplate reader (Thermo Scientific, Waltham, MA, USA). Pure recombinant ToxA was used as standard to obtain a calibration curve to determine *ToxAentero* levels in tobacco leaf tissues (expressed as µg per gram of fresh leaf tissue, µg g^{-1} FW). The background OD values from the negative control (extract from WT plant) was subtracted from those of the test extracts. Data derived from ELISA studies were analyzed by one-way ANOVA using SPSS v.19.0 software (SPSS, Richmond, VA, USA).

Immunogenicity assay

The immunogenic potential the plant-made *ToxAentero* was assessed in 12-week old female BALB/c mice as described by Rosales-Mendoza et al. (2008). Test mice groups (n = 4) were orally or subcutaneously immunized with the plant-made antigen from the transgenic line L9 (*ToxAentero* group) or the wild-type line (WT group) as negative control. For subcutaneous immunization, the test doses consisted of 10 mg of fresh tobacco leaves (containing ≈ 55 ng of *ToxAentero*) milled in 300 µL of PBS and clarified by centrifugation. Oral immunization consisted of 50 mg of the tobacco tissues (containing ≈ 275 ng of *ToxAentero*) pulverized in 300 µL of PBS and intragastrically administered. The immunization regime scheme consisted of four weekly immunizations (on days 1, 8, 15, and 22). Sera and feces samples were collected weekly for antibody content analysis.

The humoral response against *ToxAentero* was measured in serum and feces by ELISA as previously described by Rosales-Mendoza et al. (2009). Plates were coated overnight at 4 °C with pure recombinant ToxA or each peptide (LTB, ST, LptD, or FliC) using 1 µg per well and later blocked with 5% milk PBS-based solution at 25 °C for 2 h. Plates were incubated overnight at 4 °C with sera samples (1:20 to 1:160 dilutions). For feces analysis, samples were mixed in PBST supplemented with 1 mM PMSF and 5% non-fat milk, clarified by centrifugation at 16,000×g and 4 °C for 15 min, and placed in the wells (1:2 to 1:16 dilutions). Secondary labeling was performed with goat horseradish peroxidase-conjugated anti-mouse antibodies against IgG for sera or IgA for feces (1:2000 dilution, Sigma, St. Louis MO, USA) at 25 °C for 1 h. Immunodetection was revealed by adding an ABTS substrate (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic) (ABTS, Sigma, St. Louis MO, USA) plus 1 mM H_2O_2) for 60 min. OD values were measured at 405 nm in a microplate reader. Antibody titers were defined as the reciprocal of the highest sample dilution leading to a mean OD value above the corresponding value from the WT group plus 2 × SD. Data generated were analyzed by a one-way ANOVA using SPSS v.19.0 software (SPSS, Richmond, VA, USA).

Results

Design and construction of the pBI121 *ToxAentero* vector

In a previous work, epitopes reported with high immunoprotective efficacy against the target enteric pathogens were identified (Trujillo et al. 2020). Among them, the LT and ST

Fig. 2 Detection of the plant-made *ToxAentero* protein. **a** Dot Blot analysis for protein extracts from transgenic positive lines or the WT line as negative control and pure recombinant ToxA as positive control. **b** Western blot analysis for protein extracts from transgenic positive lines or the WT line as negative control and pure recombinant ToxA as positive control (13 kDa). A mouse anti-ToxA serum was used in both analyses for labeling
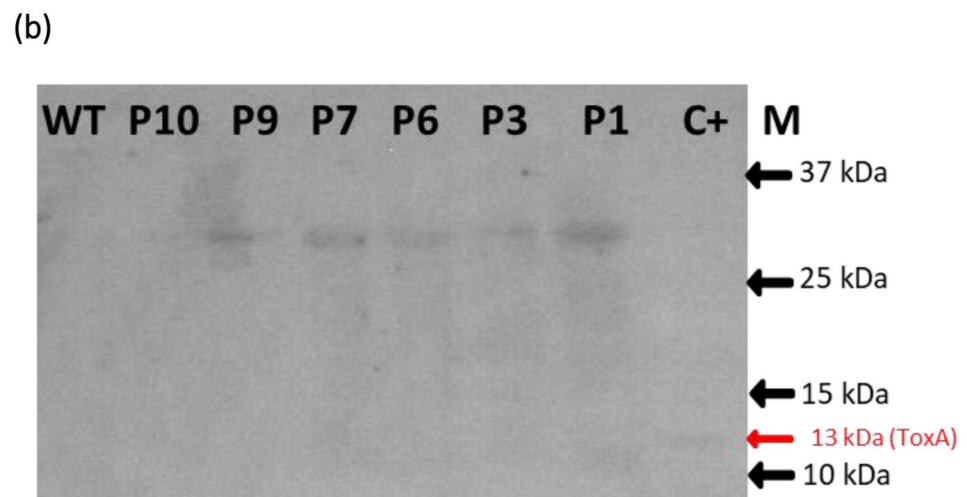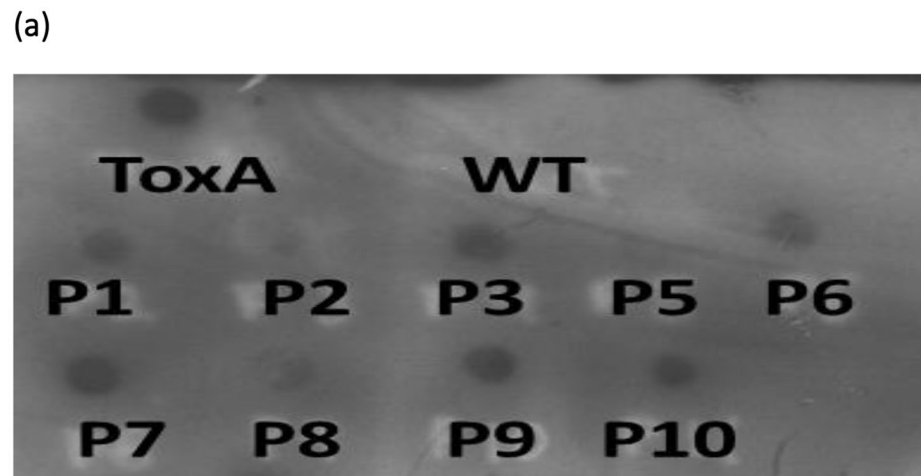

Fig. 3 Expression levels of the *ToxAentero* protein in tobacco transformed lines determined by ELISA. The quantification was performed in protein extracts from transformed plants or the WT plant as negative control. Labeling was performed with a mouse anti-ToxA serum. A calibration curve was made with pure ToxA was used to determine the recombinant protein yields. The asterisk denotes significant differences versus the WT $(P < 0.05)$
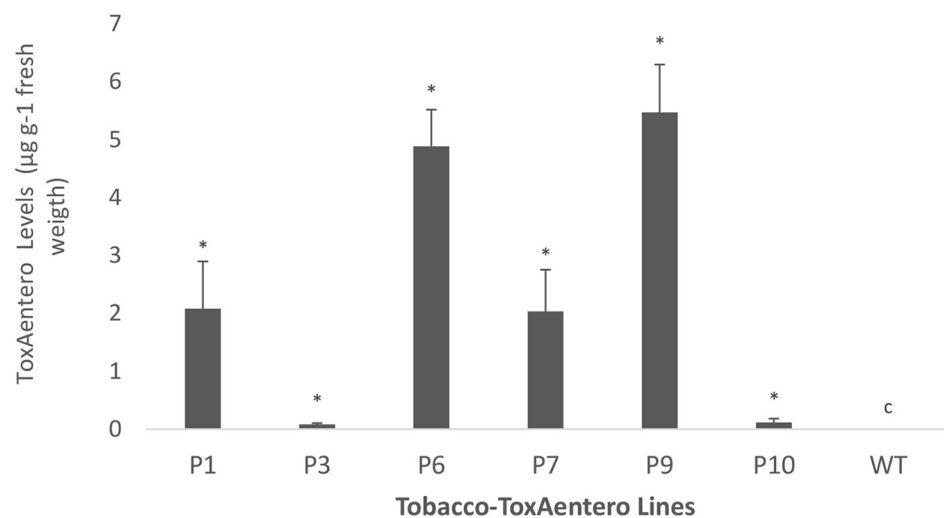
antigens from ETEC, FliC from *S. typhimurium*, and LptD from *V. parahaemolyticus* have been reported as effective immunoprotective antigens. Epitopes of these antigens were selected and used to in silico assemble the multiepitopic gene named *ToxAentero*, which was obtained by synthetic

approaches and successfully cloned into the pBI121 vector (Fig. 1a).

Presence of *ToxAentero* transgene in transformed tobacco lines

Several putative transgenic lines were obtained from the kanamycin-resistant recovered shoots after performing the standard protocol for tobacco transformation. Transgenic plants were regenerated after two months following co-cultivation; obtaining eleven independent lines (L1-L11) selected from individual explants. DNA samples from transformed lines were analyzed by PCR for the presence of the *ToxAentero* transgene (769 bp amplicon) showing a positive amplification in nine of the eleven lines, whereas the wild-type control plant sample showed no amplification (Fig. 1b).

Tobacco plants express and accumulate the *ToxAentero* recombinant protein

To confirm the presence and integrity of the recombinant protein, anti-ToxA Dot and Western blot analyses were performed; revealing the presence of a ≈ 28 kDa- immunoreactive protein in six tobacco lines. No signal was observed for the protein sample of the wild type control plant, which validates the specificity of the assay (Fig. 2). The accumulation levels of the *ToxAentero* recombinant protein in the obtained tobacco lines were determined by ELISA using anti-ToxA serum for labeling and a standard curve made with pure recombinant *E. coli*-made ToxA. The results revealed that the *ToxAentero* yields in tobacco lines ranged from 0.8 to 5.46 $\mu g\ g^{-1}$ fresh leaves weight; with lines 9 and 6 as the highest producers (Fig. 3).

Plant-made *ToxAentero* induces humoral responses in mice

Immunogenicity of the plant-made *ToxAentero* protein was assessed in terms of its capacity to induce humoral responses in BALB/c mice, which were either subcutaneously (s.c.) or orally (p.o.) immunized with the plant-made *ToxAentero* produced by the transgenic tobacco line 9. Significant anti-LT IgG levels (mean titer = 160) were observed in sera from both subcutaneously (s.c.) or orally (p.o.) immunized mice (Fig. 4a). Interestingly, the response was similar in magnitude regardless of the immunization route. The measurement of anti-LT IgA showed higher levels in feces from mice orally-immunized (mean titer = 8) compared to s.c.-immunized mice (mean titer = 4) (Fig. 5a). Concerning anti-ST antibodies, the p.o-.immunized mice showed higher IgG (mean titer = 160) (Fig. 4b) and IgA (mean titer = 16)

levels compared to the IgG (mean titer = 80) and IgA (mean titer = 2) levels from s.c.-immunized mice (Fig. 5b). In addition, similar seric anti-LptD IgG responses were induced (mean titer = 160) in mice s.c.- or p.o.-immunized with the plant-made *ToxAentero* (Fig. 4c), whereas anti-LptD IgA levels (mean titer = 2) in feces from s.c.-immunized mice were slightly lower than those for the p.o.-immunized group (mean titer = 4, Fig. 5c). Moreover, significant anti-FliC IgG systemic responses were detected at similar levels in both s.c.- or p.o.-immunized mice (mean titer = 40) (Fig. 4d). Measurements of intestinal IgA responses against FliC, measured in feces, revealed a positive response in both s.c.- or p.o.-immunized mice (mean titers of 2 and 4, respectively; Fig. 5d).

Discussion

In the present study a multiepitopic protein containing the PirA-like toxin (ToxA) as innovative adjuvant/carrier was designed and expressed in plant cells. This plant-based multiepitopic protein was designed based on antigenic determinants from the enteric pathogens ETEC, *V. cholerae*, *V. parahaemolyticus*, and *S. typhimurium* (Jacob et al. 1983, 1985; Newton et al. 1989; Bergman et al. 2005; Kremer et al. 2011; Rosales-Mendoza et al. 2011; Zha et al. 2016). The selected epitopes with known protective capacity were linked to the ToxA sequence to obtain a chimera called *ToxAentero*.

Transgenic tobacco lines carrying the *ToxAentero* gene were confirmed by PCR. Dot and Western blot analyses showed the presence of *ToxAentero* and validated its integrity according to the observed molecular weight (28 kDa). The *ToxAentero* expression levels determined by ELISA ranged from 0.8 to 5.46 $\mu g\ g^{-1}$ FW, which were similar to those reported for other multiepitopic proteins expressed in plants (Soria-Guerra et al. 2007; Rubio-Infante et al. 2015; Nieto-Gómez et al. 2019; Trujillo et al. 2020). However, the differential protein accumulation among modified lines might be associated to the transgene random insertion site into the plant tobacco genome (Kim et al. 2007). Importantly; no phenotypic alterations were observed in the modified tobacco lines, which is a key observation given the fact that other plant-expressed bacterial toxin subunits exerted toxicity in some plant species. For instance, Rawat et al. (2011) demonstrated that the expression of the Cry1Ac endotoxin has detrimental effects on both the in vitro and in vivo growth and development of cotton and tobacco transgenic plants. All the lines that showed appreciable levels of expression were found to be phenotypically abnormal.

Since alkaloids with potential toxic effects are present in tobacco plants, one important aspect to evaluate in

Fig. 4 Systemic humoral response elicited in mice upon subcutaneously (s.c.) or oral (p.o.) administration of plant-made *ToxAentero*. **a** anti-LT/CT, **b** anti-ST, **c** anti-LptD, and **d** anti-FliC IgG titers were measured by ELISA. Mice were subjected to a scheme comprising four weekly doses. Samples (sera) were taken one week after each immunization. The asterisk denotes significant differences between s.c. and p.o. routes *(P < 0.05)*
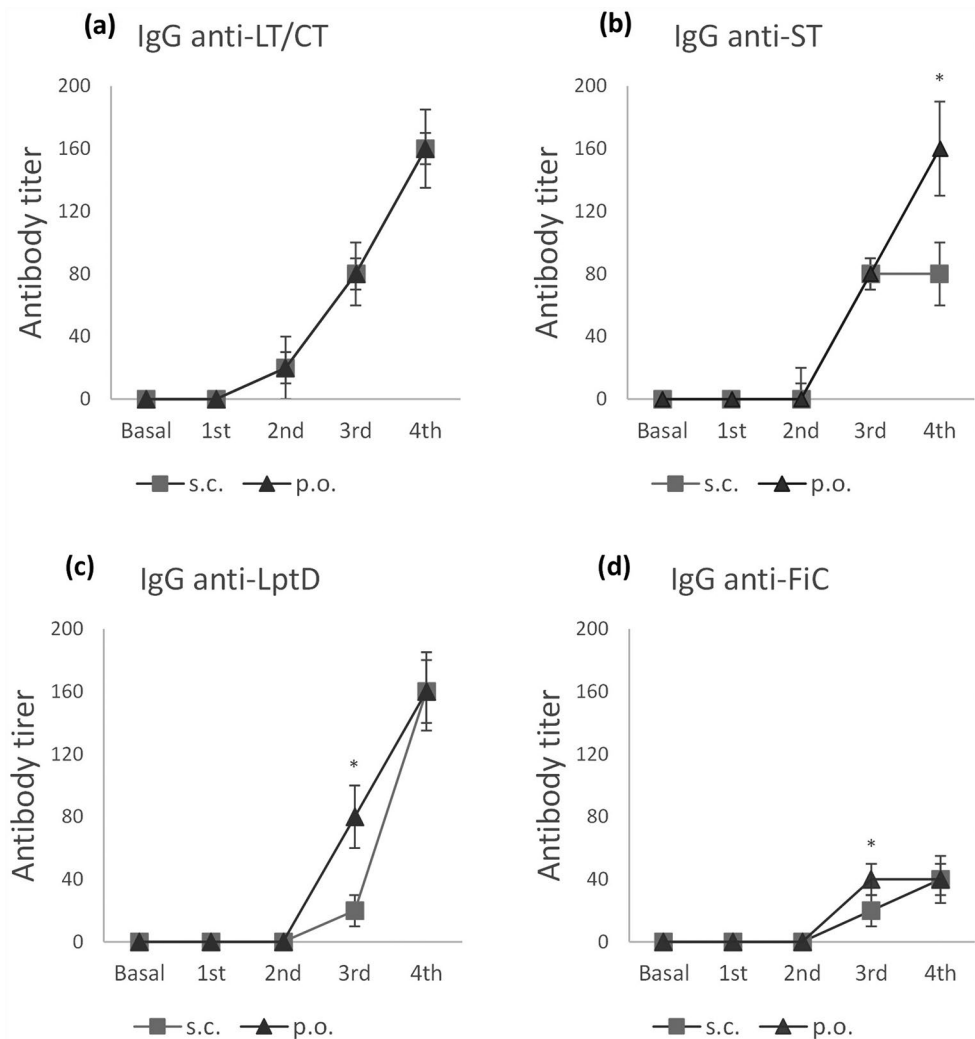

preclinical settings is the potential toxicity of the plant-based immunization scheme. The lack of obvious toxic effects in mice subjected to such schemes supports the use of tobacco for a preliminary assessment of plant-based vaccines (Rosales-Mendoza et al. 2009; Ríos-Huerta et al. 2017; Monreal-Escalante et al. 2019). A common path in the molecular farming field consists in proving the concept in tobacco and subsequently producing the target antigen in hosts that are appropriate for immunization such as edible crops (carrot, corn, and lettuce) (Appaiahgari et al. 2017; Rosales-Mendoza et al. 2017; Daniell et al. 2019; Arevalo-Villalobos et al. 2020).

The immunogenicity assessment performed in mice revealed that the plant-made *ToxAentero* is immunogenic using low doses (≈ 55 and ≈ 275 ng for s.c. and p.o. immunization, respectively). Significant specific IgG production in sera against the four epitopes (CT/LT, ST, LptD, and FliC) was observed in subcutaneously and orally immunized mice. A similar IgA response in feces was detected against the four epitopes (CT/LT, ST, LptD, and FliC) in

mice immunized by both routes. The mucosal immunogenic activity of the plant-made *ToxAentero* in terms of IgA induction was similar to that of a chimeric protein based on LTB as carrier (*LTBentero*; Trujillo et al. 2020). The immunogenic properties of *ToxAentero* are considered highly attractive due to its capacity to induce high levels of systemic IgG antibodies against CT/LT, ST, and LptD (Mean titer = 160) and significant IgG anti-FliC (Mean titer = 40). In contrast, the *LTBentero* candidate induced lower IgG responses, particularly anti-LptD IgG (Mean titer = 20), and failed to induce anti-FliC IgG responses. This might due to the particular folding of the two proteins that led to a differential display and processing of the target epitopes (Nyambi et al. 2000). Another hypothesis is that ToxA possesses higher adjuvanticity for the induction of humoral responses than LTB. Further studies are need to assess these hypotheses. Previous studies have demonstrated the high immunogenicity of recombinant ToxA and its protective potential against a *V. parahaemolyticus* challenge in fish (Pacific red snapper (*Lutjanus peru*), gilt head bream (*Sparus aurata*), and

Fig. 5 Mucosal humoral response induced in mice upon subcutaneous (s.c.) or oral (p.o.) administration of the plant-made Tox-Aentero. **a** anti-LT/CT, **b** anti-ST, **c** anti-LptD, and **d** anti-FliC IgA titers were measured by ELISA. Mice were subjected to a scheme comprising four weekly doses. Samples (feces) were taken one week after each immunization. The asterisk denotes significant differences between s.c and p.o. routes ($P < 0.05$)
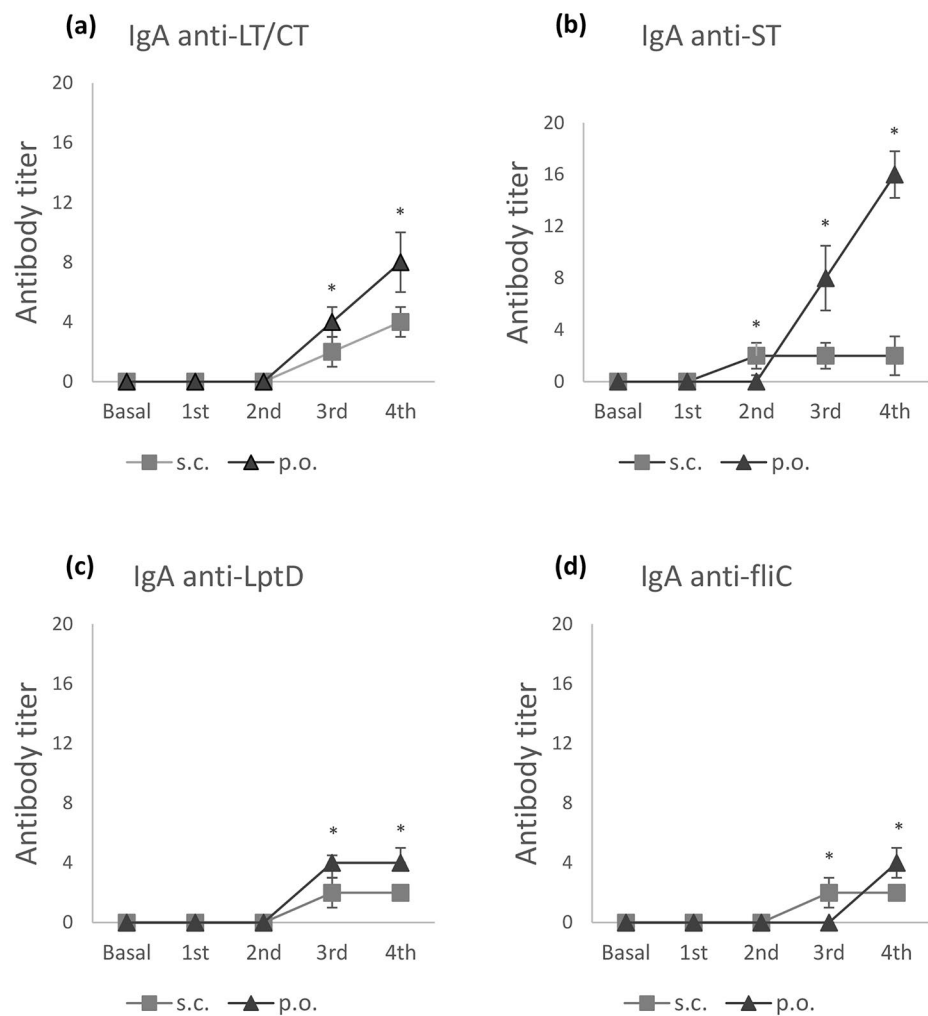
shrimp (Reyes-Becerril et al. 2016, 2017; Campa-Córdoba et al. 2017). Moreover, Monreal-Escalante et al. (2019) found strong IgG and IgA antibody induction against ToxA when mice were orally immunized with tobacco plant-based ToxA. Interestingly, this study found that the IgG levels in orally immunized mice were higher than those in s.c. immunized mice. This finding is particularly relevant to propose the oral route of administration to induce immune responses and protection against enteric pathogen infections. Mechanistic studies are envisioned to decipher the potential of ToxA as mucosal adjuvant/carrier in which comparisons against LTB should be considered as it is a well know potential mucosal adjuvant (da Hora et al. 2011; De Haan et al. 1998; Nashar et al. 2001).

Several studies have shown that epitope-based vaccines exhibit substantial advantages over conventional vaccines. However, epitope vaccines are associated with a poor immunogenicity, which can be overcome by conjugating the selected epitopes with built-in adjuvants (e.g., some carrier proteins or new biomaterials). When designing epitope-based vaccines, the following types of built-in adjuvants are typically used: (1) pattern recognition receptor ligands (i.e., toll-like receptors); (2) virus-like particles; (3) bacterial toxin proteins; and (4) novel delivery systems (e.g., self-assembled peptide nanoparticles, lipid core peptides, and polymeric or inorganic nanoparticles). (Lei et al. 2019). Although some adjuvants are currently used in vaccines licensed for human use, they are usually used as mixtures with antigens. In contrast, the use of built-in adjuvants make the vaccine formulation a simpler process and can significantly improve immunogenicity, thus offers a substantial potential to aid in the induction of a potent antigen-specific immune response (Chen et al. 2017; Rueda et al. 2017). New and efficient adjuvants for mucosal immunization are required; consequently, bacterial toxins have been explored as a potential adjuvants (i.e. LTB fron enterotoxigenic *E. coli*, CTB from *V. cholerae*, and Cry1Ac from *B. thuringiensis*; Guerrero Manriquez and Tuero 2021). The findings of this study provide evidence on the potential of ToxA as a new mucosal adjuvant and open the path for more research.

It should be considered that the design of multieptopic vaccines implies some immunological challenges; for instance (i) epitope selection, which requires a deep understanding of the pathogen's antigenic diversity, its immune evasion mechanisms, and the validation of conserved epitopes capable to elicit strong immune responses across multiple strains or variants (Lundegaard et al. 2011). (i) addressing proper immunogenicity since subunit vaccines often result in poor immunogens (Flower 2003). (iii) epitope interference, meaning that epitopes might compete for immune recognition or negatively impact their immunogenicity (Ndifon et al. 2009). (iv) immune dominance, meaning that some epitopes could dominate the immune response induced while for others a poor adaptive immune response is induced (Kedl et al. 2003). It must be pointed out that in this study significant levels of IgG and IgA against all the target epitopes were successfully induced. Future efforts will be focused on assessing other immunological parameters, such as immunoprotective effects of the vaccine candidate upon a challenge with the target enteric pathogens.

In conclusion, plant cells were transformed and able to synthesize significant levels of the functional multiepitopic protein *ToxAentero*, which triggered strong systemic (IgG) and mucosal (IgA) antibody responses against antigenic determinants from *E. coli* ETEC, *V. cholerae*, *V. parahaemolyticus*, and *S. typhimurium*, which are relevant enteric pathogens. Furthermore, PirA-like toxin from *V. parahaemolyticus* stands as a potential adjuvant for vaccines administered by either oral or parenteral routes.

Statements and Declarations

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