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Original Article

Expression of verocytotoxic *Escherichia coli* antigens in tobacco seeds and evaluation of gut immunity after oral administration in mouse model

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Verocytotoxic *Escherichia* (*E.*) *coli* strains are responsible for swine oedema disease, which is an enterotoxaemia that causes economic losses in the pig industry. The production of a vaccine for oral administration in transgenic seeds could be an efficient system to stimulate local immunity. This study was conducted to transform tobacco plants for the seed-specific expression of antigenic proteins from a porcine verocytotoxic *E. coli* strain. Parameters related to an immunological response and possible adverse effects on the oral administration of obtained tobacco seeds were evaluated in a mouse model. Tobacco was transformed *via Agrobacteium tumefaciens* with chimeric constructs containing structural parts of the major subunit FedA of the F18 adhesive fimbriae and VT2e B-subunit genes under control of a seed specific GLOB promoter. We showed that the foreign Vt2e-B and F18 genes were stably accumulated in storage tissue by the immunostaining method. In addition, Balb-C mice receiving transgenic tobacco seeds via the oral route showed a significant increase in IgA-positive plasma cell presence in tunica propria when compared to the control group with no observed adverse effects. Our findings encourage future studies focusing on swine for evaluation of the protective effects of transformed tobacco seeds against *E. coli* infection.

Keywords: *Agrobacterium tumefaciens*, edible vaccines, *Escherichia coli*, pig, verocytotoxins

Introduction

Verocytotoxic *Escherichia* (*E.*) *coli* strains, particularly the O138, O139 and O141 serogroups, are often responsible for oedema disease (OD), a serious enterotoxaemia of piglets that causes considerable economic losses [12,14,27,32]. About 70% of the affected pigs die, and surviving pigs may grow slowly. In the pathogenesis of OD, two important virulence factors are represented by the Shiga-like toxin II variant (VT2e) and the F18 adhesive fimbriae, which are often found in association [18]. Specifically, the development of OD is caused by the extra-intestinal effects of VT2e toxins, which are bipartite molecules composed of a single enzymatic, intracellularly active A-subunit and a pentamer of B-subunits associated with receptor binding [7,10,30]. The F18 adhesive fimbriae are long, flexible, filamentous structures responsible for the ability to adhere to specific receptors on porcine intestinal villi [3,20]. The backbone of the F18 fimbrial structure is composed of multiple copies of the major subunit FedA, whereas the minor adhesive subunit FedF is located at the tip [14]. Some studies have shown that intestinal colonization with live (F18-positive) *E. coli* strains resulted in significantly increased levels of anti-fimbrial antibodies, especially IgA, in the serum and intestinal wash fluids [1,25,26,29,31,34]. Pigs that have gone through the infections develop effective protection against recolonization of the intestine and have increased fimbrial antibodies in the serum. However, no vaccines are currently available and OD outbreaks require antibiotic medication, which may have negative effects on the environment and contribute to the increase of antimicrobial resistance; accordingly, the development of an effective oral vaccination strategy is of interest. In this context, plants have considerable potential and represent a promising alternative to biopharmaceutical protein

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production, as well as many practical advantages in terms of costs, safety, storage and transportation [11,28,31]. Antigens derived from various pathogens can be synthesized at high levels in their authentic forms in plant tissues, and the oral administration of plant-expressed antigens has been shown to be effective at inducing local immune responses [4,8,9,16]. The development of a mucosal vaccination system is an innovative and interesting way to avoid injections and for the production of specific antibodies in mucous membranes, where many pathogens gain access to the body [17,24]. Therefore, this study was conducted to engineer tobacco plants for the seed-specific expression of antigenic proteins as a model of edible vaccine against porcine verocytotoxic *E. coli* infection. Accordingly, we focused on virulence factors that, if lost or inactivated by specific antibodies, lead to a reduction in the pathogenicity of *E. coli*, namely the B subunit of the Shiga-like toxin and F18 fimbriae. The second objective of this study was to evaluate parameters related to local immunity, histological structure and possible adverse effects on the oral administration of tobacco seeds as an edible vaccine in the mouse model.

Materials and Methods

Isolation and evaluation of the B subunit of VT2e genes and F18

The genes encoding the Vt2e-B and FedA subunit of the F18 fimbriae were isolated from genomic DNA purified from a liquid culture of a wild type O139 *E. coli* strain by polymerase chain reaction (PCR) using the experimental conditions reported in Table 1. Oligonucleotide primers, including unique cloning sites for specific endonucleases (*BamH* I- 5′, *Sac* I-3′), were used to facilitate direct subcloning of the fragments in the plant transformation vector. The amplified Vt2e-B and F18 genes were purified from the agarose gel (Geneclean; Q-Bio Gene, Canada) and then inserted into a high copy number plasmid vector (pGEM-T easy; Promega, Italy) and used for XL1B *E. coli* strain transformation by electroporation. Preliminary clones selection was conducted by restriction analysis followed by DNA sequencing.

Construction of plant expression vectors and tobacco transformation

Two expression cassettes were designed, one for VT2e-B and another for F18. DNA *Sac*I-*BamH*I fragments obtained by digestion of pGEM-T-F18 and pGEM-T-VT2eB were separately subcloned in a pBI-GLOB vector (Patent WO0004146). GLOB, which is the soybean basic 7S globulin promoter (DDBJ accession no. AX006477), was used for the seed-specific expression of antigenic proteins according to Reggi *et al*. [19]. The chimeric constructs pBIpGLOB-F18 and pBIpGLOB-VT2eB (Fig. 1) were used to transform *Agrobacterium* (*A.*) *tumefaciens* strain EHA105 by electroporation. Tobacco leaf discs (*Nicotiana tabacum* L., *cv. Xanthi*) were transformed *via A. tumefaciens* as previously described [13].

The plants were analysed for the presence of foreign DNA using internal primers of the F18 and VT2e-B sequences by PCR and 80 ng of genomic DNA extracted from young leaves of the regenerated plants as a template according to the method described by Doyle and Doyle [6]. The mRNA was evaluated by Northern blot analysis of the immature seeds (12 days after pollination) of all PCR-positive plants. Northern blot analysis was carried out using DIG-labelled RNA probes hybridized with total RNA extracted with one volume of 50 mM Tris buffer pH 7.5 containing 150 mM NaCl, 5 mM ethylendiaminetetracetic acid (EDTA), 1% sodiumdodecilsolphate (SDS), 150 mM β-mercaptoethanol and one volume of phenol : chloroform (1 : 1). Electrophoresis, blotting and hybridization were performed as previously described [23], and specific RNA detection was conducted using CDP-star (Boehringer

Table 1. Experimental conditions used for the isolation of F18 gene and VT2e-B gene by PCR

Antigens	Oligonucleotide sequences	PCR size (pb)	PRC conditions	ACC number
F18 adhesive fimbriae	5′**ggATCC**ATgAAAAgACTAgTgTTTATTTCTTTTg 3′**gAgCTC**TTACTTgTAAgTAACCgCgTAAgC	519	25 cycles of - Den: 1 min at 94°C - Ann: 1 min 20 sec at 56°C - Ext: 1 min 30 sec at 72°C	GenBank M61713
VT2e-B subunit	5′**ggATCC**ATgAAgAAgATgTTTATAgCgg; 3′**gAgCTC**TTAgTTAAACTTCACCTgggCAA	270	25 cycles of - Den: 1 min at 95°C - Ann: 1 min at 50°C - Ext: 1 min 30 sec at 72°C	GenBank X81417

Detected genes, primers used in PCR amplification, lengths of obtained PCR products and PCR conditions. The restriction enzyme recognition sites used in cloning are shown in bold. Den: denaturation temperature, Ann: annealing temperature, Ext: extension temperature.

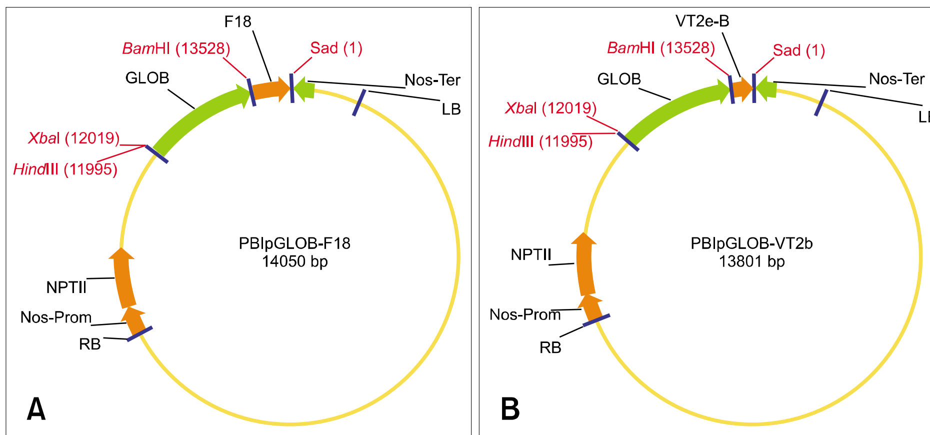


Fig. 1. Chimeric constructs used for *Agrobacterium tumefaciens* EHA105 transformations. Transgenes carrying the kanamycin resistance gene were inserted under control of the GLOB promoter and NOS terminator. (A) pBIpGLOB-VT2eB was 13800 bp. (B) pBIpGLOB-F18 was 14049 bp.

Mannheim, Germany) according to the manufacturer's instructions. The total proteins were extracted from all mature transformed tobacco lines by homogenization with liquid N_2 in a mortar and protein extraction using solubilisation buffer (50 mM Tris, pH 8, 5 mM EDTA, 200 mM NaCl, 0.1% Tween 20). Protein content was estimated by a Bradford assay (BioRad Laboratories, USA) using bovine serum albumin as the standard. The expression of VT2e-B in the total protein sample was evaluated by Western blotting with specific polyclonal antibodies obtained from New Zealand rabbits after immunization with VT2e-B expressed through the pET-system (Novagen, Germany) in BL21 *E. coli* strain. Samples (80 μg total protein) were loaded into a 10% polyacrylamide gel together with the Precision Standards (BioRad Laboratories) and a positive control of 200 ng of VT2eB protein obtained through the pET-system (Novagen) in BL21 *E. coli* strain (Fig. 2).

The proteins were transferred to an Immobilon-P^{SQ} membrane (Merk Millipore, Germany) with the Trans-Blot SD apparatus (BioRad Laboratories), after which the filters were incubated overnight with rabbit polyclonal anti-VT2e-B serum (1 : 5,000). Following incubation for 1 h with HRP-conjugated secondary antibody (1 : 10,000), the chemiluminescence was developed using a SuperSignal West Pico Trial Kit (Thermo Fisher Scientific, USA).

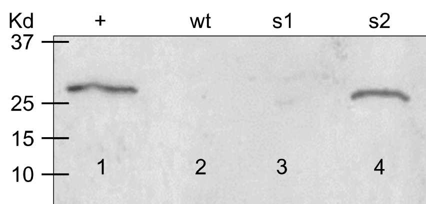


Fig. 2. Western blot of VT2-B. Lane 1: positive control represented by 200 ng of VT2e-B expressed through the pET-system in BL21 *Escherichia. coli* strain, lane 2: wild type (WT), corresponding to non-transformed tobacco seed proteins, lanes 3 and 4: samples.

The presence/absence of the F18 protein in the total soluble protein fraction from tobacco seeds (solubilisation buffer: 50 mM Tris, pH 8, 5 mM EDTA, 200 mM NaCl, 0.1% Tween 20) of F18 Northern blot positive plants was evaluated by agglutination on slides with F18+ polyclonal serum (Biovac, Israel) according to the method previously described by Chen *et al.*, with brief modification [2]. Total proteins extracted from untransformed tobacco seeds were used as a negative control. A sample of 50 L of total proteins was then placed on a slide with 50 L of F18 antibody solution and evaluated for the presence/absence of a reaction by optical microscopy (×100). In addition, a sandwich enzyme-linked immunosorbent assay (ELISA) was performed to demonstrate the expression of F18 (FedA subunit) in tobacco seeds. Briefly, the wells of a microtiter plate (Nunc Maxisorp Plate, Sigma-Aldrich) were coated with an F18-specific polyclonal antibody raised in rabbits (diluted 2,000 times in 50 mM sodium bicarbonate pH 9.4) for 2 hours at 37°C. Next, blocking was performed with PBS + 0.2% Tween80 overnight at 4°C. After washing with PBS + 0.2% Tween20, the wells were incubated with 1) F18 fimbriae [serial ½ dilution starting from 1 μg/mL diluted in ELISA dilution buffer (PBS + 0.2% Tween20 + 3% BSA)], 2) TSP extract from tobacco seeds transformed with the FedA gene or 3) TSP extract from wild type tobacco seeds for 1 h at 37°C. Thereafter, an F18-specific monoclonal antibody (IMM02) [29] was added to the wells, followed by rabbit anti-mouse-HRP (Dako, Denmark). Samples were then diluted 1/1,000 in ELISA dilution buffer and incubated for 1 hour at 37°C. Each incubation step was followed by three washing steps with PBS + 0.2% Tween20. Finally, ABTS and H_2O_2 were used as chromogen and substrate and the optical density was spectrophotometrically measured at 405 nm (OD405).

The positive producing lines were selected and

self-pollinated. The second generation was propagated in a greenhouse to produce the required amount of seed.

Oral immunization of mice

Fourteen four-week old female Balb-C mice (Harlan Laboratories, USA) were divided in two groups and placed in cages with seven mice for the treatment group (TG) and seven mice for the control group (CG). All mice were allowed to acclimatize to the animal facility for at least one week before the trial began. Tobacco seeds obtained from two independently transformed tobacco plants (F18+ and VT2e-B+) were used to prepare the treatment diets. The treatment diet, which was prepared as pellets to avoid different feed intakes, contained 10% (w/w) tobacco seeds from F18+ and 10% (w/w) tobacco seeds from VT2e-B+. Specifically, treatment diets were prepared by mixing 80 g of standard feed with 10 g of ground F18+ tobacco seeds and 10 g of ground VT2e-B+ tobacco seeds. The CG received a diet containing 20% non-transgenic tobacco seeds composed of 80 g of standard feed plus 20 g of non-transgenic tobacco seeds. The Balb-C mice were fasted for 12 h before being fed the experimental diet to increase their hunger and the likelihood of feeding. The average daily feed intake for the entire experimental period based on weighing the residual feed was 4 to 4.5 g/day/mouse; therefore, each group received 50 grams (corresponding to 7 g/day/mouse) of treatment/control diets on the days described below. The mice were fed the experimental diets *ad lib* on days 0, 5, 8, 14, 19 and 23 and the body weight and average daily gain were measured every day. All procedures were conducted in accordance with the European regulations (European Union Directive 86/609/EEC).

Immunological evaluations and micro-anatomical analyses of the intestine

Faecal samples were collected on days 13, 18 and 26. Before analysis, the samples were treated with 1.0% bovine serum albumin (BSA) and 50 mM Tris buffer (pH 7.5) for 60 min at room temperature to separate the food matrix and cellular material, after which they were centrifuged at 5,000 × *g* for 15 min. The supernatants were then used to measure the total IgA with specific ELISA kits according to the manufacturer's instructions (Cat. no. E90-103; Bethyl, Montgomery, USA). All samples were transferred to microtiter wells and analysed in duplicate at a 1 : 1,000 dilution, after which they were incubated with anti-Mouse IgA-HRP-conjugate at a working dilution of 1 : 50,000. The plates were then developed using TMB substrate and the enzymatic reaction was stopped after 20 min with 2M H_2SO_4. Finally, the plates were read at 450 nm using a microplate ELISA reader.

After 26 days, the animals were sacrificed and the entire intestinal tract was collected from each animal. Anatomical samples were excised and promptly fixed in 4% paraformaldehyde in 0.01 M phosphate buffered saline, pH 7.4, for 24 h at 4^oC, dehydrated in ethanol and then embedded in paraffin (total number of samples = 70). Three separate serial sections (100 µm apart) of each intestine sample were used, as previously described [5]. Serial microtome sections (4 µm thick) were obtained from each 100 µm-thick section and stained by Hematoxylin-Eosin (H&E) sequential staining to ascertain the structural details. The serial microtome sections of the small intestine were examined to determine the depth of intestinal crypts (C), the height of intestinal villi (V), and the V : C ratio. Serial microtome sections of the large intestine were examined to determine the depth of intestinal crypts (C; 10 per section). The glycoconjugate profile was also studied in the intestine (duodenum, jejunum, ileum, caecum and colon) using a combined histochemical method of Alcian blue 8GX pH 2.5/periodic acid Schiff (AB/PAS). Additionally, histochemical staining was performed to reveal neutral and acidic glycoconjugates. Other sections of the entire intestine were processed to visualize IgA-producing cells by immunostaining with rabbit anti-mouse IgA polyclonal antibody (Invitrogen, USA). Briefly, sections were treated with 0.05% pronase for IgA antigen retrieval before being incubated with primary antiserum diluted 1 : 200 overnight. Mouse IgA-rabbit Pab complexes were detected with a peroxidase-conjugated polymer that carried secondary antibody molecules directed against rabbit immunoglobulins (EnVisionTM+, DakoCytomation; Denmark) that were applied for 60 min. Peroxidase activity was detected with a freshly prepared solution of 10 mg 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, USA) in 0.05 M Tris-HCl buffer, pH 7.6, containing 0.03% H_2O_2. Finally, the sections were weakly counterstained with Mayer's hematoxylin, dehydrated, and permanently mounted. The specificity of the immunostaining was verified by incubating sections with: 1) PBS instead of the specific primary antibody; 2) PBS instead of the secondary antibodies. The results of these controls were negative (*i.e.* staining was abolished). The IgA-immunoreactive cell count was evaluated in the intestinal tunica propria. Quantification of IgA-immunopositive cells was based on reference to each intestinal section and extrapolated to mm^{-2} to allow comparison of the data, thus reflecting IgA-immunopositive cell density. All observations were made using an Olympus BX51 light microscope (Olympus, Japan) equipped with a digital camera and the observer was not aware of the origin of the sections.

Statistical analysis

Statistical analysis of the data was performed using the SAS statistical software (Version 8 2000; SAS Institute, USA). Data describing the performance, average daily gain and ELISA results were analysed by one-way

ANOVA using treatment as the main factor. Quantitative data from the micro-anatomical analyses were analysed by a mixed model ANOVA that included the fixed effects of the treatment and the random effects of each mouse. Values from each mouse were considered the experimental units for all response variables. Data are presented as the least squared means ± SEM. Differences between means were considered statistically significant at $p \leq 0.05$.

Results

Isolation and evaluation of F18 adhesive fimbriae and VT2e-B subunit genes

PCR products putatively encoding the B-subunit of VT2e and F18 fimbriae were identified on agarose gel (1.5～0.9%) as bands with a length of 270 and 519 basepairs, respectively. Since the inserted products showed the expected size in agarose gel, they were recovered from the gel, diafiltrated against water and ligated into plasmid T-vectors for restriction analyses and sequencing. The sequencing results of three clones of F18 and three clones of the VT2e-B subunit demonstrated the complete homology of our sequences with the sequence in the database. Additionally, the obtained data confirmed the correct insertion of genes and the homology of the sequence of obtained genes with GenBank (National Center for Biotechnology Information, USA).

Transformation of tobacco and molecular analyses of regenerated plants

The engineered vectors were used to generate a transgenic population composed of 30 and 25 (transformed for VT2e-B and F18, respectively) independently kanamycin-resistant transgenic plants, which had a similar morphological appearance to wild-type plants. About 80% of the lines of transformed tobacco plants that were screened for the presence of genes by PCR on DNA from young leaves were harbouring transgenes [21]. Samples containing transgenes were identified by the presence of an amplified product of 0.5 Kb, representing the gene encoding F18 fimbriae, or by the presence of an amplified product of 0.25 Kb, representing the gene encoding VT2e-B.

Northern blot analyses of tobacco seeds were conducted to select transcription positive transformants, verify proper mRNA processing and estimate RNA abundance depending on the position effects of the integrated transgene. Northern blot analyses showed signals corresponding to F18 mRNA (Fig. 3) in 83% of the PCR positive F18 plants and a signal corresponding to VT2e-B in 45% of the PCR positive plants.

Different lines were compared for transgene transcription, and lines with stronger signals were selected for subsequent plant generations [22]. Overall, 14 ± 1 mg of soluble proteins were extracted from samples of 100 mg of tobacco seeds. Western blot analysis was detected based on VT2e-B signals in all samples, and comparison with a positive control (VT2e-B protein expressed by the pET system in BL21 *E. coli* strain) indicated that the amount of VT2e-B per gram of seeds was approximately 0.6 mg, corresponding to 0.3% of the total soluble protein in tobacco seeds (Fig. 2). No cross-reacting proteins were identified in any of the wild-type seed extracts, and no traces of degradation products were apparent in any of the transformed samples. The presence of the major subunit of F18 fimbriae in the soluble proteins extract from F18 mRNA positive seeds was confirmed by agglutination on slides using a polyclonal F18-specific antibody (Fig. 4). Furthermore, sandwich ELISA using F18-specific antibodies revealed that small amounts of FedA were present in the total soluble protein fraction (OD405 = 0.07). No signal was obtained when the wells were incubated with TSP extracted from wild type seeds, and no signal was obtained in the uncoated wells. The amount of FedA protein present in the total soluble protein fraction was estimated to be 1 ng/mL using a concentration series of purified F18 fimbriae as a standard. The best lines of tobacco regenerated plants were selected, propagated i*n vitro* and then grown in a greenhouse.

Oral immunization and evaluation of mice

Throughout the experimental period, no mice revealed

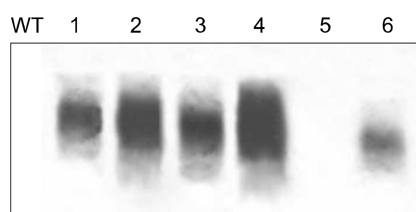


Fig. 3. Northern blot analyses for F18 mRNA detection. Lane WT: total mRNA extracted from wild-type tobacco seeds, lanes 1～4: samples positive for the presence of mRNA corresponding to F18 fimbriae, lane 5: negative sample, lane 6: positive sample.

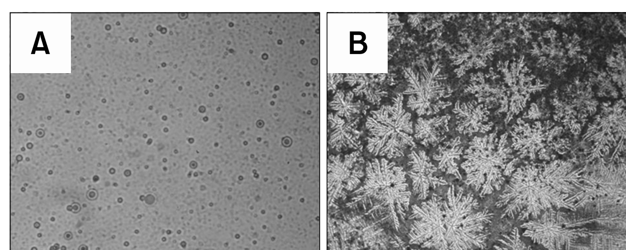


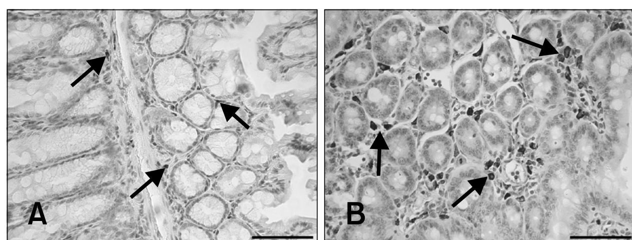
Fig. 4. Agglutination on slides with F18+ polyclonal serum. (A) Total protein extracted from wild-type seeds. (B) Total protein extracted from F18+ seeds.

enteric pathologies, and the animals' growth was determined to be normal for Balb-C mice (21 g ± 0.08 g live weight). Moreover, dry matter intake (DMI) and growth parameters (weight, average daily gain) did not differ between experimental groups, and were in agreement with the standard performance of Balb-C mice (data not shown; $p > 0.05$). Mice fed transgenic tobacco seeds had an average individual feed intake of 4.5 g; however, the highest feed intake (corresponding to 7 g/day/mouse) was observed on days in which the treatment was administered, likely as a result of the previous 12 h of fasting. In fact, no residual food was observed on the days after treatment for any each group. Based on these findings, the average estimated dose of delivered VT2e-B was about 0.42 mg/day/mouse. No adverse reactions to the tobacco seeds were observed under the experimental conditions.

Table 2. Histological and histometric evaluation of intestinal samples of mice

	CG	TG	*p* values*
Duodenum			
Villi height (μm)	167.54 ± 3.44	168.93 ± 3.32	0.758
Crypt depth (μm)	59.17 ± 2.11	63.48 ± 2.25	0.219
V : C ratio	2.83 ± 0.05	2.69 ± 0.02	0.817
Jejunum			
Villi height (μm)	150.50 ± 3.81	152.20 ± 4.20	0.544
Crypt depth (μm)	57.96 ± 1.41	58.37 ± 1.22	0.617
V : C ratio	2.59 ± 0.04	2.61 ± 0.05	0.118
Ileum			
Villi height (μm)	157.97 ± 2.24	159.66 ± 2.01	0.322
Crypt depth (μm)	58.03 ± 1.45	58.16 ± 1.35	0.411
V : C ratio	2.73 ± 0.07	2.75 ± 0.07	0.838
Caecum			
Crypt depth (μm)	65.72 ± 3.55	63.33 ± 4.58	0.365
Colon			
Crypt depth (μm)	66.22 ± 3.01	64.75 ± 3.58	0.611

Effects of tobacco seeds on villi height, crypt depth and V : C ratio within the intestine of control (CG) and tobacco seed-supplemented (TG) mice. *Values are means SEM, n = 14.



Effects of tobacco seeds on IgA-immunoreactive cells within the intestine of control (CG) and tobacco seed-supplemented (TG) mice[1]

	CG	TG	*p values*
Duodenum	493.68 ± 13.6	577.50 ± 9.9	0.039
Jejunum	333.30 ± 9.2	418.11 ± 14.5	0.031
Ilenm	129.69 ± 16.5	205.26 ± 6.6	0.027
Caecum	192.06 ± 18.7	300.30 ± 16.0	0.018
Colon	287.43 ± 14.4	416.12 ± 17.7	0.015

[1]Values are means ± SEM, n = 14

Fig. 5. Duodenum. IgA-producing cells can be seen in the intestinal tunica propria of a CG and TG mouse (A and B, respectively arrows). Scale bars = 50 μm.

ELISA revealed that faecal IgA did not different significantly between TG and CG, even though it was numerically higher in TG animals after 26 days (CG: 2.01 ± 0.8 *vs*. TG: 3.49 ± 0.9 respectively; $p = 0.213$).

Histological examination showed that oral treatment of the transgenic tobacco seeds by themselves or in association with the F18 fimbriae and VT2e-B subunit genes did not result in an altered microscopic structure of the intestines within groups. Moreover, the structural aspects of the GALT were similar in both experimental groups (Table 2). There were no statistical differences in villi height, crypt depth or V: C ratio between groups (both small and large intestines). The AB/PAS histochemistry showed that neutral glycoconjugates were abundant in the villi mucous cells, whereas acidic ones were abundant in the crypt mucous cells. No differences were observed in the mucous secretions between treated or control mice (data not shown). IgA-Immunohistochemistry showed the presence of several immunopositive cells in the tunica propria of the examined organs (Fig. 5). In addition, immunostaining of the intestine showed that dietary administration of transgenic tobacco seeds (TG) promotes a significant increase in the number of mucosal IgA-producing cells of the tunica propria in both small and large intestines.

Discussion

This study showed that foreign Vt2e-B and F18 fimbrial genes derived from a wild type verocytoxic *E. coli* strain could be stably incorporated into the tobacco plant genome via transcription through the nuclear apparatus of the plant for specific expression in the seeds, and that these genes were inherited by the next generation. As is typical of plants transformed with binary vectors *via Agrobacterium* species, integration of the pBIpGLOB/F18/NOS-T construct was variable. Dissimilar yields of amplified PCR products were obtained from different plants, suggesting different gene copy numbers and integration of the transgene into sites with variable transcription competencies. The absence of detectable PCR products in some plants, despite kanamycin selection, could reflect the development of antibiotic resistance or genetic instability after integration. The detection of transgenic proteins in

seeds demonstrated the tissue-specific expression of recombinant antigenic proteins induced by seed-specific GLOB promoter based on previously published data [18]. GLOB is a promoter of the gene coding for soybean basic 7S globulin (DDBJ accession no. AX006477), *i.e.* a seed protein synthesized in about 3% of total protein by the developing embryo and localized intracellularly in protein storage vacuoles. In the present study, this aspect is important because seeds, but not leaves, showed a low level of alkaloids and allowed the preparation of edible material for direct oral delivery for subsequent preliminary evaluations of local immunity in a mouse model. Moreover, seeds provide a stable environment for protein synthesis and storage and may also be practical as protective vehicles for transporting edible vaccines into the gut.

Notably, the FedA protein of F18 fimbriae was only estimated in low amounts and was probably underestimated. It is possible that the extraction procedure for total soluble proteins from tobacco seeds was not optimal and resulted in loss of the FedA protein. Another explanation could be the low stability of the FedA protein after extraction, resulting in a denatured, non-soluble protein, since the FedA subunit is normally part of the F18 fimbrial polymeric structure, as observed by other authors [15].

The strategy of the experimental design was to engineer tobacco plants for the expression of two antigens that would induce the production of specific mucosal antibodies with two different actions against verocytotoxic *E. coli* infections upon oral administration. At the beginning of verocytotoxic *E. coli* colonization, specific anti-adhesion antibodies induced disruption of bacterial attachment, after which anti-VT2e-B antibodies induced disruption of VT2e enzymatic activities. The development of an oral vaccination strategy through a plant-vaccine could offer an innovative and interesting method to prevent verocytotoxic *E. coli* infections and OD. Currently, the disease can only be controlled with antibiotic molecules, which are able to control bacterial growth, but not the production of toxins or the consequent lesions. Moreover, the mucosal immune system plays a pivotal role in the primary defence against pathogens by preventing binding of the microbes or their toxins to the epithelium.

Subsequent preliminary evaluation of the mucosal immunogenicity of transgenic tobacco seeds was carried out in swine. For optimal induction of mucosal immunity, the plant vaccines were delivered to the gut-associated lymphoid tissues (GALT), which are the major sites for induction of local immune responses, *via* the oral route with a specific scheme for enhancing mucosal tolerance [30,33]. No significant differences were observed upon histometrical analysis of control and treated mouse intestines (both small and large intestines), confirming that tobacco seeds did not produce detrimental effects on the intestine structure and that they could be administered orally. Immunostaining of the intestine showed that dietary administration of transgenic tobacco seeds (TG) promotes a significant increase in the number of mucosal IgA-producing cells of the tunica propria in both small and large intestines, which was not observed in the CG receiving wild type tobacco seeds. These data suggested improvement of the gut mucosal immune system and reflect the progressive increase in the local immune response actively involved in microbial infections. Faecal IgA titre did not reveal significant differences among experimental groups, which was probably due the limited number of mice included in this preliminary evaluation. Nevertheless, faecal IgA levels were higher in the treated mice than in the control group at the end of the experiment (day 26). Our preliminary findings encouraged scientific studies with the goal of evaluating whether tobacco seeds transformed for the expression of virulence factors involved in the development of oedema disease provide a promising non-invasive method of vaccinating swine *via* their feed. Plant-derived immunogens present many potential advantages related to the management of intensive livestock. Specifically, they can be administered through the oral route without having to restrain the animals, which is less stressful for the animals and reduces labour costs in terms of the multiple injections of traditional vaccines no longer being needed. We believe that the obtained data encourage subsequent evaluation of the protective effect of oral immunization of piglets with transgenic tobacco seeds expressing antigenic proteins against verocytotoxic *E. coli*.

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