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Vespa

Evaluation of antigens stability of tobacco seeds as edible vaccine against VTEC strains

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Article

Introduction

Plants have represent a promising alternative for biopharmaceutical proteins *(Ma et al., 2003; Rossi et al., 2014).* Many plant based edible vaccines have been shown to be effective in inducing local immune responses (*Rossi et al.,* 2013). Edible vaccines can activate both mucosal and systemic immunity, as they come in contact with the digestive tract lining. This dual effect would provide first-line defense against pathogens invading through the mucosa. The antigens are released in the intestines are taken up by M cells that are present over the Payer's patches (in the ileum) and the gut associated lymphoid tissue (GALT). Edible vaccines represent an important worldwide goal for the prevention of the enteric diseases, also in livestock. In particular, the enteric infections are a significant clinical problem in pigs. Verocytotoxic *Escherichia (E.) coli* strains are responsible for serious enterotoxaemia that causes important economic losses in the pig industry. The production of a vaccine for oral administration of transgenic seeds could be a practical and efficient system to prevent the infection and to reduce the antibiotic use. This study was focused on tobacco plants, previously transformed by agroinfection for the seed-specific expression of antigenic proteins (F18 adhesive fimbriae and the B subunit of the Vt2e toxin) as model of edible vaccines against *verocytotoxic E. coli* strains. The dietary administration of transgenic tobacco seeds promotes a significant increase in the number of mucosal IgA-producing cells of the tunica propria in both small and large intestine in mice (*Rossi et al., 2013).* A protective effect of oral administration of transgenic tobacco seeds was also observed against verocytotoxic *Escherichia coli* infection in piglets *(Rossi et al., 2014).* The aim of this study was to assess the seed-expression stability, that is a important requirement in the vaccine production, of F 18 and Vt2e-B heterologous genes into the progeny of transformed tobacco plants.

Material and Methods

Production of transgenic seeds.

Seeds of control (not transformed *Nicotiana tabacum plants*), F18 transformed plants (F18+) and VT2e-B transformed plants (VT2e-B+) were seeded in pots (3 seeds/pots) containing commercial soil and placed in Fitotron® growth chambers with 200 µmol photons m^{-2} s^{-2} illuminated, with a vapor growth lamp at 25 ± 2 C° and a photoperiod of 14h light and 10h night. The R3 generation was propagated in a greenhouse (80 plants for each line) protected by a polyethylene monofilament at The Orto Botanico "G.E. Ghirardi" of the University of Milan. During the growing period all the plants were regularly watered and fertilized in same conditions.

Evaluation of seeds

Seeds of R3 generation were collected from each plant and evaluated as described below. The plants were analyzed for the presence of foreign DNA using PCR, (primers and conditions are described in table 1.) The total proteins were obtained from all mature transformed tobacco seeds by homogenization with liquid N_2 in a mortar with the solubilization buffer (50 mM Tris, pH 8, 5 mM EDTA, 200 mM NaCl, 0.1% Tween 20). Protein content was

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estimated by a Bradford assay (BioRad, Hercules, USA) using bovine serum albumin as standard. The specific antibodies were obtained from rabbit polyclonal anti-Vt2e-B serum and f-18 Peptide Polyclonal Antibody (GenScript, USA). The F18 and Vt2eB proteins were purified and used as positive controls. The F18 fimbrae was purified as described by *Goddeeris et al. (2002)* from O138 *verocitotoxic E.coli* strain.

Table 1. *Table of the oligonucleotides sequences for F18 and Vt2e-B detected by PCR.*

Gene	Oligonucleotide Sequences	PCR size (pb)	PCR conditions
F18 adhesive fimbriae	5' ggatcc atgaaaagactagtgtttatttcttttg 3' cgaatgcgccaatgaatgttcatt ctcgag	519	den. 95°C -1'; ann. 56°C -1'20";ext. 72°C -1'30"; 35 cycles
VT2e-B subunit	5' ggatccatgaagaagatgtttatagcgg 3' aacgggtccacttcaaatgattctcgag	270	den 95°C -1'; ann 50°C 1' 20"; ext 72°C 1'30"; 35 cycles

The VT2e-B protein were expressed by pET-system (Novagen) in *E.coli* BL21 (DE3) as described by Rossi et al. (2014). A competitive indirect ELISA was developed to quantify the Vte2-B and F18 proteins in tobacco seeds. Polyvinyl microtiter plates (Thermo Scientific) were coated overnight at 4°C with 100 µl of the respective purified protein (F18 and Vte2B). The same protein purified, used for coating plates, was used to make a standard curve. Plates were blocked with 100 µl/well of blocking buffer (2% BSA in PBS/tween 20) for 3 hours at 37°C. 100 µl of each standard solution and extracted samples were dispensed into the individual sterile tubes. 100 µl of diluted primary antibody (antiF18 1:15.000; anti-VT2eB 1.10.000) were added to each tube and mix accurately (30 min of incubation at room temperature RT). The reaction was transferred (100 µl) into the wells of coated plate. After a 30 min incubation at RT, the reaction was transferred into the plate. After four washing with washing buffer (PBST), 100 µl of peroxide-conjugated goat anti-rabbit (Sigma) were added to the wells and incubated at 37° C for 30 minutes. The plates were then washed four times with PBST and wells 50 µl of TMB (Sigma) were added in the wells. The reaction was stopped after 15 minutes by adding 150 µl of stopping buffer (0,4M HCl). Optical density (OD) was measured at 450 nm using a microplate reader (Biorad).

Results and Discussion

The two lines of R3 generation of Nicotiana tabacum transformed for E.coli antigenic proteins, the F18 adhesive fimbriae and the B subunit of verocytoxin, maintained the transgenes into plant genome. No cross-pollination was observed between the two transgenic lines as observed in figure 1.

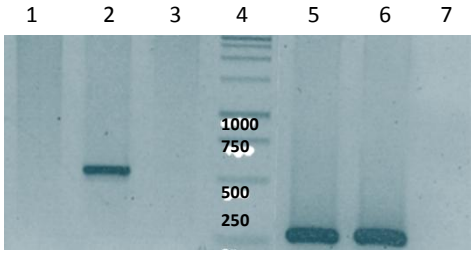

Figure 1: *electrophoresis of PCR products obtained using f18 oligonucleotides (samples 1, 2, 3) and Vt2eB oligonucleotides (samples 5,6,7). Lane 1: Genomic DNA from wild type Nicotiana tabacum; lane 2: genomic DNA from F18 positive Nicotiana tabacum; lane 3: genomic DNA from Vt2e-B positive Nicotiana Tabacum plants. Lane 4: marker (pb); lane 5: genomic DNA from Vt2e-B positive; lane 6: positive control; lane 7: genomic DNA from wild*

The total soluble proteins extracted from 100 mg of seeds obtained from F18 positive plants were about 1,8 ug/ul, were estimated using Bradford assay. F18 proteins was estimated about 66-74 ng/100 mg of tobacco seeds, by the developed ELISA system. The total soluble proteins extracted from 100 mg of seeds obtained from VT2e-B positive plants were about 2.2 µg/ µL, evaluated with Bradford assay. VTE2-B proteins was estimated about 340-370/100 mg of tobacco seeds, using ELISA assay. The different amount of antigenic proteins in the two transgenic lines is due the *Agrobacterium*-mediated transformation. In fact, *Agrobacterium tumefaciens* binary vector system is an efficient tool to transform plant cells; however, the exogenous gene integrates at semi-random into the nuclear chromosomes.

Conclusions

Obtained data demonstrated that the genes coding for VT2e-B and the F18, represent important antigens and virulence factors of *E. coli*, therefore can be stably incorporated into the next generation of *Nicotiana tabacum* genome. The foreign VT2e-B and F18 adhesive fimbriae genes , derived from verocytotoxic *Escherichia coli* strain,

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stably incorporated into tobacco genome under control of seed-specific promoter, were well transcribed through the nuclear apparatus of the plant for specific expression in seed

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