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TECHNICAL REPORT

Induction of a protective antibody response to FMDV in mice following oral immunization with transgenic *Stylosanthes* spp. as a feedstuff additive

Dong Mei Wang · Jian Bo Zhu · Ming Peng · Peng Zhou

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Abstract The expression of antigens in transgenic plants has increasingly been used as an alternative to the classical methodologies for the development of experimental vaccines, and it remains one of the real challenges in this field to use transgenic plant-based vaccines effectively as feedstuff additives. We report herein the development of a new oral immunization system for foot and mouth disease with the structural protein VP1 of the foot and mouth disease virus (FMDV) produced in transgenic *Stylosanthes guianensis* cv. Reyan II. The transgenic plantlets were identified by polymerase chain reaction (PCR), Southern blotting, and northern blotting; and the production of VP1 protein in transgenic plants was confirmed and quantified by western blotting and enzyme-linked immunosorbent assays (ELISA). Six transformed lines were obtained, and the level of the expressed protein was 0.1–0.5% total soluble protein (TSP). Mice that were orally immunized using studded feedstuff mixed with desiccated powder of the transgenic plants developed a virus-specific immune response to the structural VP1 and intact FMDV particles. To our knowledge, this is the first report of transgenic plants expressing the antigen protein of FMDV as feedstuff additives that has demonstrated the induction of a protective systemic antibody response in animals. These results support the feasibility of producing edible vaccines from transgenic forage plants, and provide proof of the possibility of using plant-based vaccines as feedstuff additives.

Keywords FMDV · *Stylosanthes guianensis* cv. Reyan II · Transgenic plant · Edible vaccines

D. M. Wang (✉) · M. Peng · P. Zhou
State Key Laboratory of Tropical Crop Biotechnology, Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Science, Haikou 571101, China
e-mail: aweeo@tom.com

P. Zhou
e-mail: zhp6301@126.com

J. B. Zhu
Key Laboratory of Agriculture Biotechnology of Shihezi University, Shihezi, Xinjiang 832003, China

Introduction

Foot and mouth disease virus (FMDV) is the causative agent of an economically important disease affecting meat-producing animals (Visser and Thiel 2001). Although comprehensive vaccination of all susceptible hosts, using inactivated virus as the immunogen, constitutes the basis of all sanitary plans for the control and eradication of the disease, there are some disadvantages associated with the production and the application of vaccines. It is always desirable to find a new, secure, low-cost way of obtaining vaccines.

Transgenic plants that overexpress relevant antigens have increasingly been used for the production

of experimental immunogens (Arakawa et al. 1998; Carrillo et al. 1998; Haq et al. 1995; Mason et al. 1992; Mason et al. 1998; Wigdorovitz et al. 1999; Ritcher et al. 2000; Yu and Langridge 2001; Walmsley et al. 2003; Sojikul et al. 2003; Varsani et al. 2003). Compared to the traditional approach, these transgenic plants are potentially inexpensive sources of antigen since they could be used as edible vaccines, and more interestingly, in the form of feedstuff additives. The FMDV structural protein VP1 that carries critical epitope antibodies has been reported to be overexpressed in *Arabidopsis thaliana*, alfalfa, and potato. The VP1 protein produced in these transgenic plants has been successfully used as an experimental immunogen for eliciting a virus-specific protective antibody response after parenteral or oral administration (Carillo et al. 1998; Wigdorovitz et al. 1999; Carrillo et al. 2001). In all those cases, immunity, however, seemed to be relatively poor and could be further improved. A careful choice of plant host system could also be important for improving immunity and reducing cost.

Stylosanthes spp. is an important commercial forage legume in tropical areas worldwide. In light of its high crude protein content *Stylosanthes* spp. could be considered as an ideal host system of animal plant-based vaccines. In this study, we utilized VP1 of FMDV serotype O1 Campos (O1C) as a model to investigate the possibility of using transgenic *Stylosanthes* spp. plants as a source of antigens in the production of experimental feed additives. The results presented herein demonstrate that mice that were orally immunized with the transgenic plants as feedstuff additives developed a virus-specific immune response.

Materials and methods

Plasmids and reagents

E. coli strain DH5α, *E. coli* strain BL21 (DE3), *Agrobacterium tumefaciens* strain LBA4404, pET (30a), and pBI121 were provided by the State Key Laboratory of Tropical Crop Biotechnology. Goat anti-rabbit IgG-AP was the product of the Sino-American Biotechnology Company, China. All the restrictive enzymes and the plasmid pGEM-T were products of Promega Corporation. rTaq DNA polymerase is the product of TaKaRa company (Da Lian, China). The plasmid pTVP1 that contains the sequence of the VP1 gene was stored by our laboratory.

Plant and medium

The seeds of *Stylosanthes guianensis* cv. Reyan II were kindly provided by the Pasturage Research Center, China Academy of Tropical Agricultural Science. The seeds were incubated consecutively on MS medium to obtain sterile plantlets (Jiang et al. 1998). Luria-Bertani (LB) culture medium was prepared as described by Sambrook et al. (1989).

Plant tissue culture medium contained 3% sucrose and 0.75% agar, pH 5.6–6.2. Conditions of incubation for *Stylosanthes* spp. were an illumination intensity of 144–180 μmol m^{-2} s^{-1}, 14 h/day, Tm 28 ± 1°C.

Production of transgenic plants of *Stylosanthes guianensis* cv. Reyan II containing the VP1 gene

A 654 bp DNA fragment encoding the VP1 gene was amplified by PCR from the plasmid pGEM-T. This VP1 DNA fragment, after being digested with SpeI and Sac, was cloned into a binary vector, pBI121, behind a cauliflower mosaic virus (CaMV 35S) promoter. The newly created plasmid was called pBIVP1. pBIVP1 was then introduced into *Agrobacterium tumefaciens* strain LBA 4404 by electroporation following the procedure described by Wen-Jun and Forde (1989).

To produce transgenic plants, cotyledons of sterile young plants of *Stylosanthes guianensis* cv. Reyan II were cut into about 0.25 cm^2 pieces, co-cultivated with the *Agrobacterium tumefaciens* for 1 day, and were then placed on selection culture medium. The selection for transgenic plantlets was performed using 50 mg/l of kanamycin according to the published procedures (Jiang et al. 1998).

Genetic analysis of the transformed plants

PCR analysis

The presence of *VP1* in the kanamycin-resistant plants was detected by PCR analysis. Total DNA was extracted from approximately 0.1 g of leaves with CTAB according to the standard protocol (Sambrook et al. 1989). The PCR reactions were carried out with a forward primer 5′-TCGAGCTCTCTT TACAAG-3′ and a reverse primer 5′-GTACTAAGTATGGCT

ACCTCC-3, which specifically amplified a 654 bp DNA fragment of the *VP1* gene. PCR reactions were performed in a 25 μl final volume, containing 100 ng plant DNA, 0.25 μl rTaq DNA Polymerase (3000U), 2.5 mM $MgCl_2$, 100 μM of dNTPs, and 0.5 μM of each primer at 94°C/30 s, 41°C/30 s, 72°C/60 s, for 30 cycles. The products of PCR were subjected to electrophoresis on a 1% agarose gel and then visualized with ethidium bromide. The plasmid pBIVP1 was used as the template for the positive control.

Southern and northern blotting analyses

In order to identify the integration of the *VP1* gene into the genome of *Stylosanthes* spp., Southern blotting assays were conducted using the digoxygenin (DIG) DNA Labeling and Detection Kit of Boehringer Mannheim (Roche). Genomic DNA was extracted with phenol-chloroform, ethanol precipitated, and dissolved in Tris EDTA (TE) buffer. The total DNA was digested with *Sac*I and *EcoR*I overnight. DNA fragments were separated through a 1.0% agarose gel in Tris-borate-EDTA (TBE) buffer, blotted onto a Hybond-N^+ membrane (Roche) overnight, and ultraviolet (UV) cross-linked (Stratalinker, Stratagene). The membrane was hybridized with DIG-labelled DNA probes (654 bp of the *VP1* gene). Conditions for hybridization and washes were the same as described by Church and Gilbert (1984). After being washed the membrane was incubated with an alkaline-phosphatase-labeled anti-mouse IgG rabbit antiserum for 1 h at 37°C. Then the reaction was developed by the addition of the substrate Nitro blue tetrazolium/5-Bromo-4-chloro-3-indolyl phosphate (NBT/BCIP).

To perform northern blotting analysis, total RNA was isolated from the leaves of transformed plants by guanidinium thioisocyanate extraction. The amount of RNA was estimated by measuring OD_{260}. About 200 μg total RNA was transformed to a nylon membrane. The probe preparation and method of hybridization were the same as for Southern blotting.

Antibody production and quantification of VP1 protein in transgenic plants

Recombinant VP1 protein and antibody production

The full length of the *VP1* gene was amplified by PCR from the plasmid pGEM-T and then inserted between the *Nde*I and *Xho*I sites of a pET(30a) expression vector. This expression plasmid was transformed into lysogenic *E. coli* strain BL21 (DE3) cells according to the standard procedure (Sambrook et al. 1989). *E. coli* colonies were selected for the confirmation of *VP1* gene by restriction enzyme digestion analysis and DNA sequencing. A positive clone was cultured in liquid LB medium at 37°C. VP1 protein expression was induced by adding 1 mM isopropylthio-*β*-galactoside (IPTG). Cells were harvested 3 h after induction. The expression screening result showed that the VP1 protein mainly existed in the inclusion bodies. The inclusion bodies were isolated and dissolved in 8 M urea and PBS buffer. The VP1 protein was purified with Ni^+-appetency pole (ProBond Purification System). To generate anti-VP1 polyclonal antibodies, the purified VP1 protein was emulsified in an equal volume of Freund's complete adjuvant and injected intradermically on a rabbit at multiple sites.

Western blotting assay

To evaluate the antigenicity of the expressed protein, western blotting assay was performed. Soluble proteins from crude plant extract were separated electrically on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred onto a Polyvinylidene Fluoride (PVDF) membrane. The membrane blot was probed with the antiserum against the FMDV VP1 in a 1:80 dilution, followed by probing with alkaline phosphatase conjugated with a goat anti-rabbit IgG in a 1:1000 dilution. The signal was detected by colorimetric reaction with NBT/BCIP as substrates.

ELISA assay

Total soluble proteins (TSP) of tubers were extracted in PBS buffer, and the ELISA assay of the VP1 protein in transformed plants was conducted. The wells of a microtiter plate were coated with total soluble proteins from the wild type, the transformed plants, and the known FMDV VP1 standard protein, respectively, at 4°C overnight. After three washes with PBST (PBS containing 0.05% Tween 20), the plate was incubated with a rabbit anti-FMDV VP1 antiserum in a 1:120 dilution, and then alkaline phosphatase conjugated with a goat anti-rabbit IgG in

a 1:2000 dilution. The color reaction was initiated by adding *p*-nitrophenyl phosphate (pNPP) substrate and terminated by the introduction of 2 N H_2SO_4. The plate was read at a wavelength of 450 nm in a MR 500 microplate reader (Dynatech). Quantitative analysis of total soluble proteins was conducted according to the methods described by Bradford (1976).

Analysis of antibody induction in response to the feeding of transgenic proteins

Feedstuff preparation

After western blot and ELISA analysis, the transformed line in which the highest VP1 expression level was identified was named HGVP1. HGVP1 and nontransformed lines were grown in a greenhouse and harvested after 3 months. The whole plant materials were dried in an oven at 45°C overnight, and later pulverized (0.45 kg hay meal being obtained from 1 kg). The powders were mixed as: 120 mg hay meal, 2 g starch, and some honey, and the mixture was kneaded into granules by hand.

Induction of immune response in mice orally immunized with feedstuff prepared from transgenic plant hay meals

Eighteen adult (60- to 90-day-old) male Balb/c mice were divided into three groups: the positive control group, the negative control group, and the testing group. In the positive control group, each mouse was parenterally immunized each time with 80 μg of pure FMDV VP1 protein (emulsified in Freund's adjuvant with a total volume of 0.4 ml). In the testing group and the negative control group, each mouse was fed each time with the feedstuff granules containing 120 mg hay meal of the transformed or nontransformed line, respectively, 2 g starch, and some honey. All of these mice were immunized orally on days 0, 15, 30, and 45.

Blood was collected from tail veins of the mice before immunization and 10 days after each immunization to test for the presence of anti-FMDV-specific antibodies by ELISA. ELISA was performed using the antigen of either the known VP1 protein or inactivated complete FMDV particles as described by Wigdorovitz and co-workers (1999). In the former case, the VP1 protein was directly adsorbed to the plate at a concentration of 20 μg/ml. In the latter case, the inactive and complete FMDV serotype O1 Campos (O1C) particles were added to the plate at a concentration of 1.5 μg/ml afterwards. The antigen incubation was performed at 4°C overnight. The plates were washed with PBST buffer and a pool of mouse anti-VP1 antiserum was added and incubated for 1 h at 37°C. The plates were washed again and incubated for 1 h at 37°C with alkaline-phosphatase-labeled goat anti-mouse IgG. After three washes, the reaction was developed by the addition of BCIP/NBT in citrate buffer, pH 9.6. The plates were read at 450 nm with a MR 500 microplate reader (Dynatech) 30 min later. Mouse sera from the last blood collection were tested in a fivefold dilution series in blocking buffer. OD value was expressed as the mean OD ± standard deviations (SD) of sera from all the experimental mice. Serum titers were expressed as the $\log_{10}$ of the reciprocal of the highest serum dilution that gave OD readings above the mean OD ± SD of sera from six animals immunized with nontransformed lines.

Results

PCR analysis of transformed plants

Sixty different transgenic plants, from ten individual transformation events, with the ability to grow on the kanamycin medium were produced. The presence of the *VP1* gene in the transgenic plants was analyzed by PCR. Plant DNA was isolated from cell extracts and PCR reaction was performed with a pair of primers that specifically amplify a 654 bp *VP1* fragment as described in the "Materials and Methods" section. In total, 27 *VP1*-positive transgenic *Stylosanthes* spp. lines were identified; they were assigned the numbers V1–V27.

Southern blotting assay and northern blotting assay of the VP1 gene

The selective *VP1*-positive lines (V3–V12) identified by PCR analysis were investigated by Southern blotting assays. Figure 1 represents the results of the assay, showing that a DNA fragment with 1.8 kb was detected in all ten transgenic *Stylosanthes* spp. lines. This 1.8 kb DNA fragment was not detected in the wild-type *Stylosanthes* spp. The results suggested

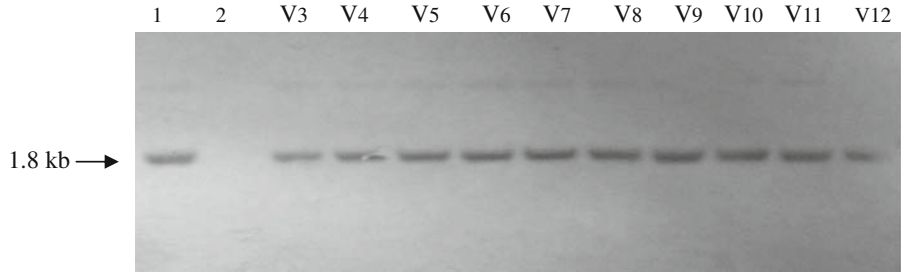

Fig. 1 Southern blotting assay of *Stylosanthes* spp. plants. A 1.8 kb DNA band was detected when the blot with total DNA was hybridized with the DIG-labeled *VP1* probe. The hybridization was conducted with the DIG-labeled *VP1* probe as described in the "Materials and Methods" section. Lane 1: pBIVP1 plasmid digested with *Sac*I and *EcoR*I as the positive control; lane 2: wild type; lanes V3–V12: transformed lines

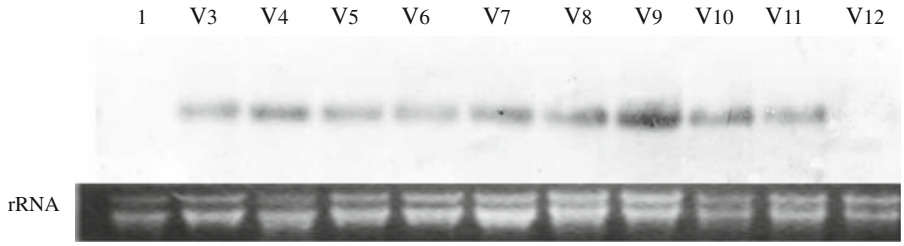

Fig. 2 Northern blotting assays of transgenic *Stylosanthes* spp. plants. An RNA band was detected when the blot with total RNA was hybridized with the DIG-labeled *VP1* probe. Lane 1: wild type; lanes V3–V12, the transgenic lines

that the transformed *Stylosanthes* spp. genome contained the inserted *VP1* gene.

To make sure that the VP1 gene in transgenic *Stylosanthes* spp. was transcribed, the ten transgenic lines (V3–V12) were selected for northern blotting analysis. The result showed that the *VP1* gene was transcribed in nine transgenic lines (V3–V11). No VP1 transcript was detected in line V12, indicating either very low transcription or possible gene silencing (Fig. 2).

Western blotting assay of the VP1 protein

To check the expression of the VP1 protein in the transgenic *Stylosanthes* spp. tubers, V3–V8 were analyzed by western blotting assay (Fig. 3). The assay revealed that a 25 kDa polypeptide, the expected molecular size of the VP1 protein, was specifically recognized by the anti-FMDV VP1 antibody. This 25 kDa polypeptide only appeared in the transgenic lines V3, V4, V5, and V7, but not in V6, V8, and the wild-type tubers. It is possible that VP1 protein expression in some transgenic lines such as V6 and V8 was too low to be detected by the western blotting assay.

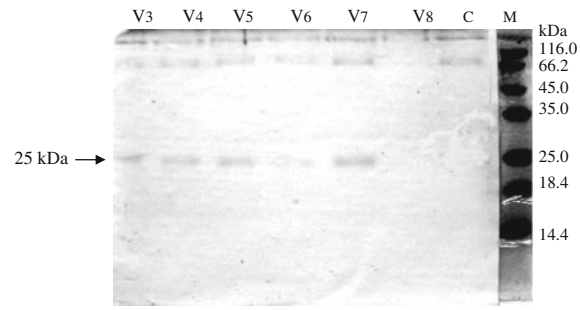

Fig. 3 Western blotting assay of VP1 protein expression in *Stylosanthes* spp. transformants. The 25 kDa VP1 protein was detected by anti-FMDV VP1 polyclonal antibodies and goat anti-rabbit IgG-AP. Lane M: the protein marker; lanes 3–8: total proteins from the transformed lines; lane C: total protein from the wild

ELISA analysis of VP1 protein

To quantitatively estimate the amount of VP1 protein in transgenic plants, ELISA analysis was conducted with transgenic *Stylosanthes* spp. and wild-type tubers. The purified VP1 protein with known concentration was used as a standard. Four transgenic lines (V3–V5 and V7) were analyzed. The results showed that the VP1 protein expression level was

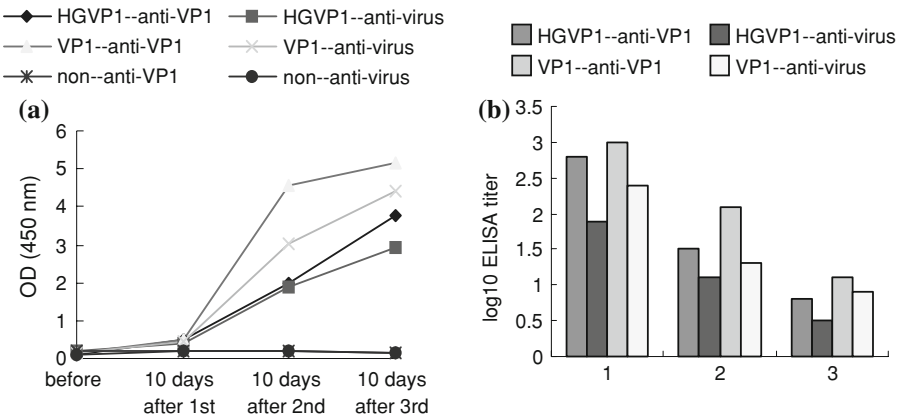

Fig. 4 Quantitative measurements of anti-VP1 and anti-FMDV particle antibodies in mice orally immunized with pBIVP1 transformed and the wild-type plant materials or in mice intraperitoneally immunized with the VP1 protein, using the ELISA method. Data represent the mean of six experimental mice immunized. (**a**) Time course of antibody development. (**b**) Titer determination of antibodies after three immunizations. The numbers at the bottom of the bars indicate different dilutions of 1:5, 1:25, 1:125. The icons at the top of figures, HGVP1-anti-VP1, and HGVP1-anti-virus, stand for the immune responses against the known VP1 protein and the inactive and complete FMDV serotype O1 Campos (O1C) particles from the sera of mice immunized orally with the transgenic materials, respectively; VP1-anti-VP1 and VP1-anti-virus stand for the immune responses against the known protein VP1 and the inactive and complete FMDV serotype O1 Campos (O1C) particles from the sera of mice immunized intraperitoneally with the purified VP1 protein, respectively

0.1–0.5% TSP. The expression level of the protein VP1 was the highest (0.5%) in V7.

Analysis of antibody response to the plant-expressed VP1

The adult Balb/c mice oral test results showed that the mice orally immunized with the feedstuff granules containing the transgenic plant hay meal from line V7 in which the protein VP1 was expressed at the highest level produced significant levels of anti-VP1 or anti-virus antibodies (Fig. 4a and b). After the second oral immunization the level of the specific antibody began to rise; the highest serum dilution was 1:125 (Fig. 4b). The immune activity obtained by oral immunization was almost comparable with that obtained by parenteral immunization. The mice fed with feedstuff granules containing the wild-type plant hay meal had little immune activity (Fig. 4a).

Discussion

A few research laboratories have reported success in using transgenic plants for FMDV antigen production (Carillo et al. 1998; Wigdorovitz et al. 1999; Carrillo et al. 2001). Although the transgenic approach appears to be a very attractive alternative to the classic methodology for producing antigens, many issues still need to be addressed. One of the disadvantages of this approach is that there is large variation among plants as hosts to produce reasonable amount of antigens, and at the same time, different plants might have different capabilities to produce the same amount of antigens with distinct immunogenic activities, which could induce different protective antibody responses. *Stylosanthes* spp. is one of the most economically important tropical forage legumes, and it has advantages such as high crude protein content (14.7%) and good resistance to dry and grazing growth conditions. In this study, we chose *Stylosanthes* spp., which proved to be an excellent host for producing FMDV antigen.

Previously, other research groups have shown that animals orally immunized with freshly harvested transgenic leaves or other tissues, or intraperitoneally immunized with transgenic leaf extract could produce certain protective antibodies (Carillo et al. 1998; Wigdorovitz et al. 1999; Carrillo et al. 2001), but there are very limited reports about transgenic plant vaccines as feedstuff additives. In this study, we explore the probability of whether the VP1 protein produced in transgenic *Stylosanthes* spp. could serve as a feedstuff additive to induce anti-VP1 antibody production when fed to mice. Our result showed that

oral immunization using the transgenic hay meal induced a strong antibody response. In our study, honey was used as one of the additives to the feedstuff, and we found this to be important for strong protective antibody induction; further investigation is needed to identify its real role.

Currently, seven immunologically different serotypes of FMDV are known (Oliveira et al. 2005; Sun and Wang 2004; Pérez Filgueira et al. 2000). A vaccine that could defend against multiple serotypes of FMDV is much needed. Knowing the critical epitopes of FMDV, a few research groups have carried out research and reported limited success in achieving good immune response activities against multiple serotypes of FMDV using mixotope peptide-based vaccine produced in transgenic plants (Dus Santos et al. 2002; Dus Santos and Wigdorovitz 2005a; Dus Santos and Wigdorovitz 2005b). These studies opened a new avenue for the development of mixotope peptide-based vaccine using transgenic plants, but much work remains to be done for further improvements. The results from our current work prove the feasibility of using transgenic *Stylosanthes* spp. as a novel and safe system for oral vaccine production against FMDV. It will be interesting to see whether transgenic *Stylosanthes* spp. can serve as an ideal host system for the production of mixotope peptide-based vaccines in the future.

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