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A novel methodology to develop a foot and mouth disease virus (FMDV) peptide-based vaccine in transgenic plants

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Abstract

The expression of antigens in transgenic plants has been increasingly used as an alternative to the classical methodologies for antigen expression in the development of experimental vaccines. However, an important limitation in most cases is the low concentration of the recombinant antigens in the plant tissues, which reduces the possibilities of practical applications. Because the site of insertion of the transferred DNA into the cellular chromosomal DNA is at random, different levels of foreign protein expression in independent transformants is expected. Strategies to allow the evaluation of a high number of the transgenic individuals, usually an expensive and very time consuming process, would permit the selection of those plants presenting the highest levels of recombinant protein expression. Here, we present the development of an experimental immunogen based in the expression of a highly immunogenic epitope from foot and mouth disease virus (FMDV) fused to the glucuronidase (*gus* A) reporter gene, which allows selection of the transgenic plants by the ß-glucuronidase (ßGUS) enzymatic activity. We produced transgenic plants of alfalfa expressing the immunogenic site between amino acid residues 135–160 of structural protein VP1 (VP135–160), fused to the ßGUS protein. Plants expressing the highest levels of the immunogenic epitope VP135–160, analyzed by Western blot, were efficiently selected based on their levels of ßGUS enzymatic activity. The FMDV epitope expressed in plants was highly immunogenic in mice which developed, after immunization, a strong anti-FMDV antibody response against a synthetic peptide representing the region VP135–160, to native virus VP1, and to purified FMDV particles. Additionally, these mice were completely protected against experimental challenge with the virulent virus. To our knowledge, this constitutes the first report of a peptide-based vaccine produced in transgenic plants that induces a protective immune response when used in experimental hosts. Also, these results demonstrated the possibility of using a novel and simple methodology for obtaining transgenic plants expressing high levels of foreign immunogenic epitopes, which could be directly applied in the development of plant-based vaccines.

Keywords: FMDV; Recombinant plants; Vaccination; Antigen expression

1. Introduction

The use of transgenic plants for the expression of relevant antigens has been increasingly used for the production of experimental immunogens [1–3,5–12]. They are, potentially, an inexpensive source of antigens that could be parenterally administered or, more interestingly, to be used as edible vaccines [1,6,7,9,11,12].

With that objective, we have been using foot and mouth disease virus (FMDV), the causative agent of an economically important disease affecting meat producing animals, as a model to investigate the possibility of vaccine production using transgenic plants as vectors. We have previously reported that the FMDV structural protein VP1, which carries critical epitopes responsible for the induction of protective neutralizing antibodies [13], could be successfully expressed as an immunogenic antigen in *Arabidopsis thaliana* alfalfa and potato and used, as experimental immunogen, for eliciting a virus-specific protective antibody response when parenterally or orally administered [2,12,14]. Nevertheless, in all those cases, the concentration of the expressed protein in the transgenic plant tissues was relatively poor. Both, the difficulty in detecting the foreign protein in the plant

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extract by Western blot, as well as the necessity of numerous immunizations in order to induce a significant immune response, indicate the low level of the expressed protein.

Thus, although the expression of immunogenic antigens in transgenic plants appears to be a very promising alternative to other methodologies for the production of recombinant proteins, its main disadvantage consists of the low concentration reached by the foreign protein in the plant tissues. This point became particularly relevant in those cases where the plant extracts are expected to be used without any further processing. Thus, increase in the concentration of the foreign protein in the transgenic plants becomes a critical issue to be considered. Among other strategies, which included genetic manipulations, an alternative to solve this problem could be the feasibility of identifying those transgenic individuals expressing exceptionally high levels of the recombinant protein. For this purpose, it would be necessary to develop methodologies that allow the screening of a large number of individual plants leading to the identification and selection of those expressing the highest levels of the transgenic protein.

In this study we present the development of a methodology based in the construction of a fusion protein composed of a very well known and easily detectable reporter gene, glucuronidase (*gus* A), fused to an epitope of interest, the antigenic determinants comprised by amino acid residues 135–160 from the structural protein VP1 of FMDV (VP135–160). The results obtained demonstrated that a large number of individuals can be readily screened by their ß-glucuronidase (ßGUS) enzymatic activity which correlates with the levels of VP135–160 expression. Mice immunized using the selected plants readily developed a strong and protective antibody response against virulent FMDV in experimental hosts.

2. Material and methods

2.1. Production of transgenic plants of alfalfa containing the VP–ßGUS gene

A 96 bp DNA fragment (VP135–160), encoding for the area between residues 135 and 160 of VP1 FMDV O1Campos (O1C) [15], was obtained by hybridizing complementary synthetic nucleotides. The native VP135–160 O1C DNA sequence was adapted to the more frequent *A. thaliana* plant codon usage. The sequence of the positive stranded synthetic nucleotide used was: 5′-TTCAGTCTAGAGGATCCATGAGATCTCTAGAAAC-GCTGTTCCTAACGTTAGAGGAGATCTTCAGGTTCTT-GCTCAAAAGGTTGCTAGAACTCTTCCTGGATCCCC-GGGTGGTT-3′. In addition to the sequence encoding residues 135–160 of VP1, the VP135–160 DNA fragment also contains, on the 5′-end, an ATG codon and the restriction sites for *Xba*I and *Bam*HI and, on the 3′-end, restriction sites for *Bam*HI and *Sma*I. The VP135–160 DNA fragment, after being digested with *Xba*I and *Sma*I, was cloned in the plasmid pBI221 (Clontech) which contains the *gus* A gene (downstream of the *Xba*I and *Sma*I restriction sites) under the control of the cauliflower mosaic virus (CaMV35S) promoter. The plasmid (pBI221VP–ßGUS) encodes the designed recombinant product, VP–ßGUS, a fusion protein containing at its amino terminus the amino acid residues 135–160 of FMDV VP1 followed by the complete amino acid sequence of the *gus* A gene. The DNA cassette containing *nptII* gene, the CaMV35S promoter and the *VP–ßGUS* gene was excised from pBI221VP–ßGUS by digesting with *Hin*dIII and *Eco*RI and re-cloned in the binary plasmid pBI121 (Clontech), previously digested in the same way in order to remove its original CaMV35S–*gus* A cassette.

The obtained recombinant binary vector, pBI121VP–ßGUS (Fig. 1A), was introduced into *Agrobacterium tumefaciens*, strain LBA 4404 by electroporation using the procedure described by Wen-Jun and Forde [16]. Petioles and embryos of alfalfa clone C2–3, kindly provided by McKersie and Bowley (Plant Biotechnology Division, Department of Plant Agriculture, University of Guelph, Canada) were co-cultivated with *A. tumefaciens* and cultured in vitro as described by Shetty and McKersie [17]. The in vitro selection was performed using 50 mg/l of kanamycin as described by McKersie et al. [18].

2.2. Genetic analysis of the recombinant plants

The presence of the recombinant gene in the kanamycin resistant plants was detected by PCR [2]. Total nucleic acids were extracted from samples of approximately 50 mg of leaves following the protocol described in Klimyuk et al. [19]. The following pair of primers: forward primer 5′-TTCAGTCTAGAGGATCCATGAGAT ACTCTAGAA-3′; reverse primer 5′-GAACCACCCGGGG-ATCCA-3′ were used to specifically amplify a 115 bp DNA fragment of the *VP–ßGUS* gene. PCR reactions were performed in a 50 ul final reaction volume, containing 100 ng of plant DNA, 2.5 mM $MgCl_2$, 100 uM of dNTPs and 0.5 uM of each primer.

2.3. Detection of the ßGUS activity in the transgenic plants

The expression of ßGUS in the plant tissues was quantified by fluorometry as described by Gallagher [20]. Briefly, 100 mg of plant tissue were ground in the presence of liquid nitrogen and the resulting macerated resuspended in GUS extraction buffer (50 mM Na phosphate, pH 7, 10 mM ßME, 10 mM Na_2EDTA, pH 8, 0.1% l-lauryl sarcosine, 0.1% Triton X-100). After clarification, 5 ug of protein were dissolved in 400 ul of GUS reaction buffer (GUS extraction buffer containing 1 mM of 4-methil-umbeliperil-ß-D-glucuronide-MUG) and incubated at 37 °C. The reaction was stopped at different times by the addition of 0.1 M

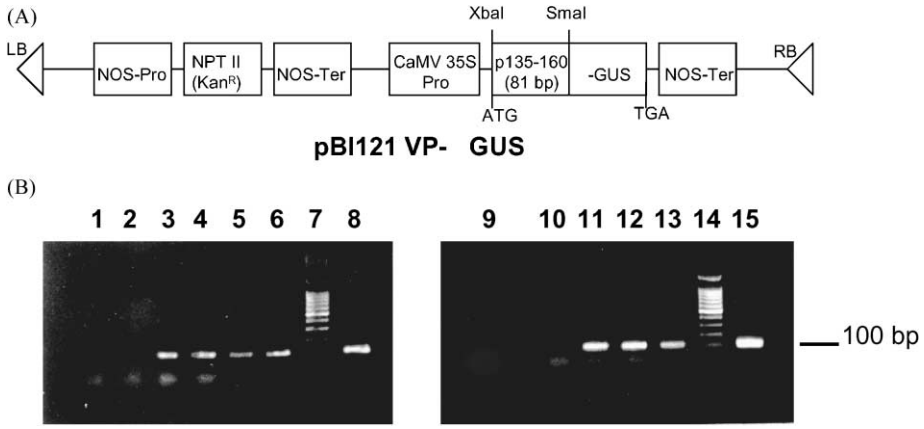

Fig. 1. (A) Schematic representation of the pBI121VP–ßGUS plasmid. (B) Detection of the *VP–ßGUS* gene in the transgenic plants using PCR. Plant DNA was isolated from cell extracts and PCR was performed with a pair of primers which specifically amplify a DNA fragments of 115 bp. Lane description: non-template (1–9), DNA from plants transformed with a non-related gene (2–10), DNA from pBI121VP–*gus* A transformed plant (3–6 and 11–13), MW markers (7 and 14) and pBI221VP–ßGUS plasmid as positive control (8 and 15).

Na_2CO_3 and read in a flourometer against a standard concentration curve constructed using different concentrations of 4-metil-umbeliferone (MU). The results are expressed as nanogram of MU produced per minute per milligram total protein in the leaf extracts of the transgenic plants compared with the standard curve. The assay was independently repeated at least twice with each of the tested plants.

2.4. Antigenic analysis of the recombinant plants

The expression of the VP135–160 epitope in the transgenic plants was analyzed by Western blot. Plant extracts were prepared by grinding 100 mg of freshly harvested plant tissue in the presence of liquid nitrogen and the resulting powder resuspended in SDS-PAGE sample buffer (50 mM Tris, pH 7.5, 1 mM PMSF, 8 M urea, 1% SDS, 2 mM DTT and 2% 2-ßME), boiled 10 min, electrophoresed in 12.5% SDS-PAGE and blotted to an Immobilon P (Millipore) membrane. The membrane was blocked overnight with PBS containing 0.05 Tween-20 (PBST), 3% skim milk (all subsequent steps were performed using this buffer) and incubated with a mouse serum raised against the synthetic peptide p135–160 for 2 h at 37 °C. The membrane was then washed using PBST, and incubated with an alkaline phosphatase labeled anti-mouse Ig goat antiserum (Dakkopats) for 1 h at 37 °C. After washing three times, the reaction was developed by the addition of the substrate NBT/BCIP (Sigma).

Quantification of the VP135–160 epitope expressed in cell extracts of the transgenic plants was also performed by Western blot using the anti-p135–160 mouse antiserum. The density of the VP–ßGUS band was directly compared with known concentrations of purified FMDV particles. In order to standardize the conditions, all FMDV particle samples were diluted in an equivalent amount of plant tissue obtained from a non-related transgenic plants.

2.5. Analysis of antibody response to the plant-expressed VP135–160 epitope

Mouse sera were evaluated for the presence of anti-FMDV-specific antibodies by ELISA and Western blot.

2.5.1. ELISA

It was performed using as antigen either a synthetic peptide (p135–160), which represents the amino acid residues of the VP135–160 epitope, or complete FMDV particles. The assay was performed exactly as described by Wigdorovitz et al. [12]. In the case of p135–160, peptide was directly adsorbed to the plate at a concentration of 15 ug/ml. To detect anti-virus particle activity, purified FMDV particles (produced, inactivated and purified as described in Berinstein et al. [21]) were added to the plate (at a concentration of 1 ug/ml) after the capturing antibody was absorbed. In either case, antigen incubation was performed for 1 h at 37 °C. Mouse sera were tested in a four-fold dilution series in blocking buffer. Serum titers are expressed as the log of the reciprocal of the highest serum dilution which gives OD readings above the mean OD + 3 SD of sera from five animals immunized with non-transformed plants.

2.5.2. Western blot

Inactivated purified FMDV was resuspended in sample buffer. The electrophoresis and blotting as well as the blocking and washing steps was performed as already described for the detection of the VP135–160 epitope in the plant tissues. Then, the membrane was incubated with the corresponding mouse sera (diluted 1/20) for 2 h at 37 °C, washed using PBST, and incubated with an alkaline phosphatase labeled anti-mouse Ig rabbit antiserum (Dakkopats) for 1 h at 37 °C. After washing three times the reaction was developed by the addition of the substrate NBT/BCIP.

2.6. Challenge experiments

Mice were challenged i.p. with 10^4 suckling mouse lethal doses ($SM_{50}LD$). Protection was determined by absence of viremia in the challenged mice at 36 h post-infection (p.i.). Viremia was tested by i.m. inoculation of 50 ul of a 1/10 dilution of peripheral blood to a 5–6 days old litter of six mice per blood sample [2,4,12,14,15].

3. Results

3.1. Production and genetic analysis of transformed plants

After five individual transformation events using *A. tumefaciens* transformed with pBI121VP–ßGUS, 51 different transgenic plants with the ability of growing in the presence kanamycin, were produced. The presence of the *VP–ßGUS* gene in the transgenic plants was detected by PCR. Plant DNA was isolated from cell extracts and PCR was performed with a pair of primers which specifically amplify a DNA fragments from the *VP–ßGUS* gene of 115 bp. The results obtained demonstrated the presence of an amplified product of the expected size in all the kanamycin-resistant plants tested, which was consistently absent in non-transformed plants or in plants transformed with a non-related gene (E183L from African swine fever virus, ASFV) (Fig. 1B).

3.2. Analysis of the ßGUS activity in the VP–ßGUS transgenic plants

The 51 transgenic individuals produced, harboring the *VP–ßGUS* gene, were tested for their ßGUS activity by fluorometry as described in Section 2. Among all the analyzed plants it was possible to observe a complete variety in the range of ßGUS activity, from plants showing basal values to those presenting the highest activity (Fig. 2A). The results demonstrated that approximately 5% of the tested individuals presented a remarkably high ßGUS activity. Those plants presenting the highest level of ßGUS activity (plants no. 5, 11, 12 and 29) were selected for analyzing the presence of an association between the levels of enzymatic activity and the expression of the FMDV epitopes residing in the VP–ßGUS fusion protein.

3.3. Detection of the VP135–160 epitope expression in the selected transgenic plants

The four plants selected by their ßGUS activity (plants no. 5, 11, 12 and 29) were analyzed, by Western blot using an anti-p135–160-specific mouse antiserum, for their ability to express the VP135–160 epitope. The results indicated that, coincidentally with the degree of ßGUS activity in each of the tested plants, those individuals having the highest ßGUS expression (plants no. 5 and 29) also showed the highest levels of VP135–160 peptide accumulation, presenting a unique distinctive band with the expected molecular weight for the VP–ßGUS protein (approximately 69 kDa) (Fig. 2B). The approximate concentration of the recombinant VP–ßGUS expressed in the transgenic line no. 5 was estimated to range between 0.5 and 1 mg/g of total soluble protein, based on the extinction of the signal in Western blot compared with a known control preparation using quantified purified FMDV particles (data not shown).

3.4. Induction of a FMDV-specific immune response in mice intraperitoneally immunized with plant extracts expressing VP–ßGUS

The immunogenicity of the VP135–160 epitopes expressed in the recombinant plants was tested by immunizing mice with crude extracts from plant line no. 5. Adult (60–90 days old) male BALB/c mice were immunized intraperitoneally on days 0 and 25 with plant tissue extracts from either transgenic plant expressing VP–ßGUS or from transgenic plants expressing an unrelated foreign gene (200 ul of leaf extract, containing approximately 100 mg of fresh leaf tissue, in incomplete Freund's adjuvant per animal per injection). Ten days after the last inoculation, animals were bled and the sera analyzed for the presence of anti-FMDV antibodies by ELISA and Western blot.

The results indicated that all the sera from the VP–ßGUS immunized mice presented a specific antibody response against both FMDV antigens: VP1, as demonstrated by their reactivity to the synthetic peptide p135–160, and to purified FMDV particles (Fig. 3A).

The anti-VP1 specificity of the antibody response in the sera of the vaccinated mice was further confirmed by Western blot using purified inactivated FMDV as antigen. The sera of the mice immunized with the VP–ßGUS plant extracts were tested as pools of two individual sera. The results indicated that all pools of sera specifically recognized a protein with the same relative mobility as the one recognized by a control serum, raised against the synthetic peptide p135–160 (Fig. 3B). No reactivity was detected either in ELISA or Western blot when sera from animals immunized using tissue extracts from plants expressing a non-related antigen (E183L) were used as control.

3.5. Protection of the VP–ßGUS immunized mice against the virus challenge

To test the effectiveness of the induced immune response in preventing infection following virus exposure, the vaccinated mice were experimentally challenged with infectious FMDV. The VP–ßGUS immunized mice were inoculated i.p. with 10^4 $SM_{50}LD$ of FMDV O1C and 36 h later the absence of viremia was considered as an indicator of protection [2,4,12,14,15]. The challenge experiment demonstrated 100% of protection in the VP–ßGUS immunized mice

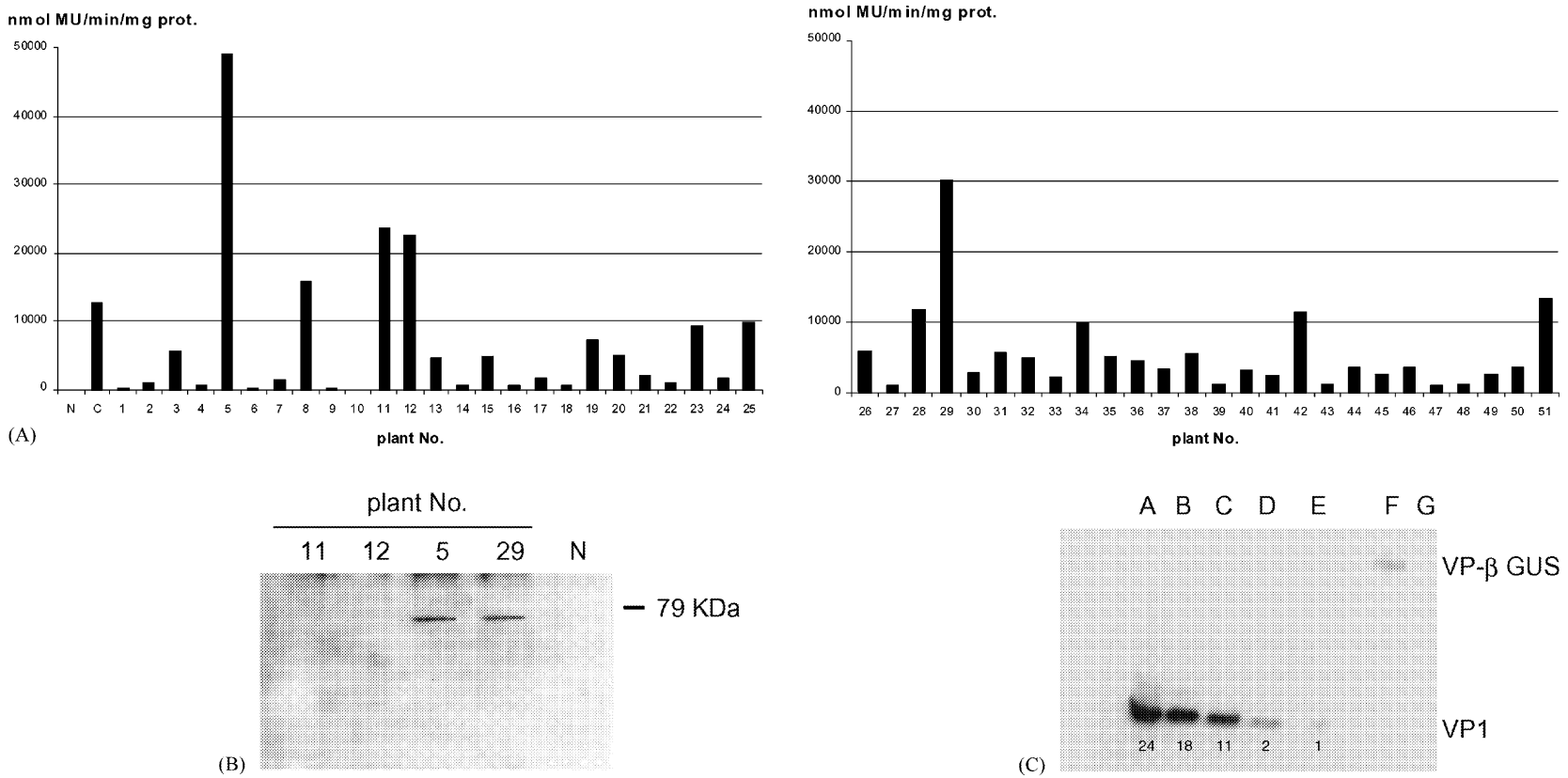

Fig. 2. (A) Detection of ßGUS activity in the pBI121VP–ßGUS transgenic plants. Leaf extracts from different individuals were tested for their enzymatic activity as described in the text. Each bar in the histogram represents the activity of individual transgenic plants (which is identified by the numbers at the bottom of the bars). Activity is expressed as nanogram of 4-metil-umbeliferone (MU) produced/min/mg of total protein. N: plants transformed with a non-related gene. C: plant transformed with pBI121. (B) Detection of the VP–ßGUS expression in selected transgenic plants by Western blot analysis. Leaf extracts from four different individuals (no. 5, 11, 12 and 29) were analyzed using a mouse antiserum specific for p135–160. N: extracts from a plant transformed with a non-related gene. (C) Western blot assay used for quantification of the VP 135–160 epitope. Lanes A–E contain 24, 18, 11, 2 and 1 ng of the epitope 135–160% in VP1 of purified FMDV particles, respectively. Lanes F and G contain 2 ug of total soluble protein extracted from plant no. 5 and an unrelated transgenic plant, respectively.

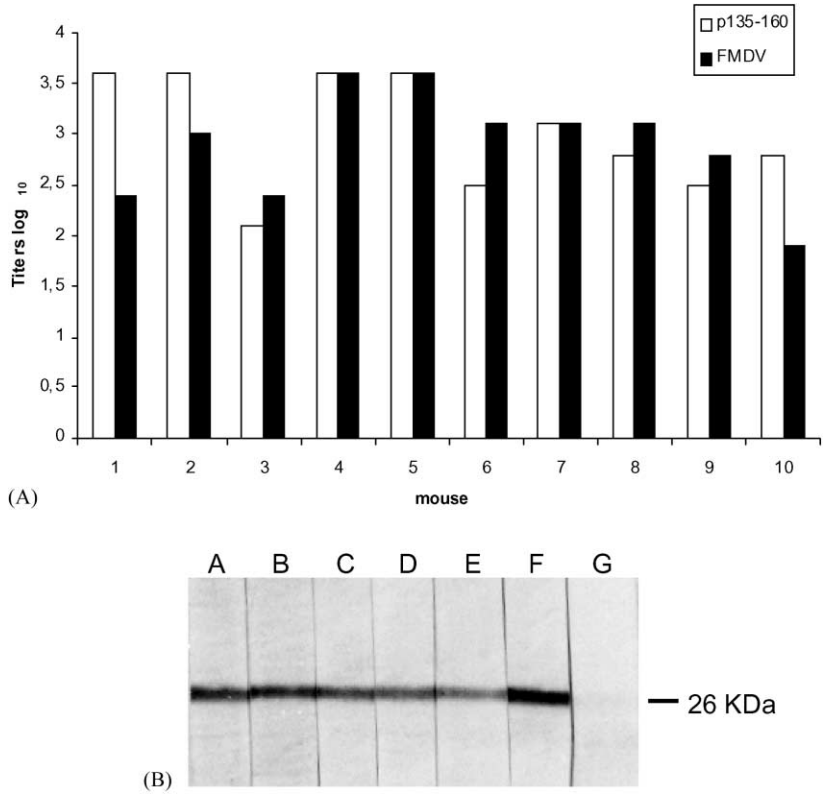

Fig. 3. Detection of antibodies to FMDV antigens in mice intraperitoneally immunized with leaf extracts from pBI121VP–ßGUS transformed plants using (A) ELISA, and (B) Western blot with purified FMDV as antigen. (A) Anti-p135–160 (empty bars) and anti-FMDV particles (filled bars) antibodies. Numbers at the bottom of the bars correspond to different mice immunized with plant line No. 5. Serum titers are expressed as the log of the reciprocal of the highest serum dilution which gives OD readings above the mean OD + 3 SD of sera from five animals immunized with plants transformed with a non-related foreign gene. (B) Lane description: different pools of sera from mice immunized with pBI121VP–ßGUS transformed plants (A–E) or from transformed with a non-related recombinant gene (G). A pool of sera from p135–160 immunized animals is used as control (F).

Table 1
Protection against FMDV challenge in mice i.p. immunized with different plant extracts

Mice immunized i.p.	Protection rate[a]
pBI121VP–ßGUS plant extracts	10/10 (100%)
pBI121 plant extracts	0/10 (0%)
Non-transformed plant extracts	0/10 (0%)
Mock immunized	0/8 (0%)

[a] Protection is expressed as a number of protected mice per number of challenged mice.

compared with the complete lack of protection in the animals immunized with extracts from either non-transformed plants or transformed with a non-related gene (Table 1).

4. Discussion

Since first described by Mason et al. [8], the use transgenic plants for antigen production had received further experimental support from several other groups who had reported the expression of different antigens using this methodology [1–3,5–11]. Although it appears to be a very promising alternative to other methods for expressing recombinant protein, the main disadvantage with this system consists in the low concentration of the antigen expressed. Some of the approaches successfully assayed to increase this expression included the use of specific reticulum retention signals sequences [1–3,5–7], the increase of the translational efficiency of the RNA using the 5′-untranslated region of the tobacco etch virus [9] or the utilization of plant-optimized synthetic genes [10]. Alternatively, the results presented here constitute a completely novel methodology which allows the production of transgenic plants expressing high levels of recombinant proteins harboring immunodominant viral epitopes which could be used for the production of plant-based vaccines.

Thus, the levels of VP–ßGUS expressed in the plant line no. 5 are, approximately, 10 times higher than those observed in transgenic alfalfa plants, which we previously developed, expressing other recombinant proteins cloned in similar binary plasmids [12]. In concordance with this, the antibody titers reached by the immunized mice were markedly higher that those previously obtained in animals receiving complete VP1 expressed in alfalfa plants, which were not

selected by their ability to produce high levels of the foreign protein [2].

Importantly, the expression levels of VP–ßGUS appears very stable during the life time of a determinate plant as well as during the asexual reproduction cycles. Thus, the expression level of plant no. 5 remained practically unchanged when monitored during 26 month experimental period (data not shown). Additionally, no significant variation was observed in the expression levels of VP–ßGUS in a study performed on 14 individuals obtained by vegetative reproduction of plant no. 5 (data nor shown). The FMDV epitopes included in VP–ßGUS, expressed in transgenic plants as the VP–ßGUS fusion protein, presented the same effectiveness, in terms of inducing a protective antibody response, as when administered as synthetic peptide [15].

We believe these results add strong support to the possibilities of using transgenic plants as a novel and safe system for vaccine production. This methodology would also potentiate the use of more sophisticated binary vectors that, by the inclusion of specific promoters, enhancers or signal sequences, leads to increased levels of expression of the recombinant genes. To our knowledge, this report would constitute the first example of a peptide-based vaccine produced in transgenic plants which induces a complete protection in experimental hosts.

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References

[1] Arakawa T, Chong DKX, Langridge WHR. Efficacy of a food plant-based oral cholera toxin B subunit vaccine. Nature Biotech 1998;16:292–7.

[2] Carrillo C, Wigdorovitz A, Oliveros JC, Zamorano PI, Sadir AM, Gómez N, et al. Protective immune response to foot and mouth disease virus with VP1 expressed in transgenic plants. J Virol 1998;72:1688–90.

[3] Castañón S, Marín MS, Martín Alonso JM, Boga JA, Casais R, Humara JM, et al. Immunization with potato plants expressing VP60 proteins protects against rabbit hemorragic disease virus. J Virol 1999;73:4452–5.

[4] Fernandez F, Borca MV, Sadir A, Fondevila N, Mayo J, Schudel A. Foot and mouth disease virus (FMDV) experimental infection: susceptibility and immune response of adult mice. Vet Microbiol 1986;12:15.

[5] Gómez N, Carrillo C, Parra F, Salinas J, Borca MV, Escribano JM. Expression of immunogenic glycoprotein-*S*-polypeptides from transmissible gastroenteritis coronavirus in transgenic plants. Virology 1998;249:352–8.

[6] Gómez N, Wigdorovitz A, Castañón A, Gil F, Ordas R, Borca MV, et al. Oral immunogenicity of the plant derived spike protein from swine-transmissible gastroenteritis coronavirus. Arch Virol 2000;145:1725–32.

[7] Haq TA, Mason HS, Clements JD, Arntzen CJ. Oral immunization with recombinant bacterial antigen produced in transgenic plants. Science 1995;268:714–6.

[8] Mason HS, Lam DMK, Arntzen CJ. Expression of hepatitis B surface antigen in transgenic plants. Proc Natl Acad Sci USA 1992;89:11745–9.

[9] Mason HS, Ball JM, Shi JJ, Jiang X, Estes MK, Arntzen CJ. Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. Proc Natl Acad Sci USA 1996;93:5335–40.

[10] Mason HS, Haq TA, Clements JD, Arntzen CJ. Edible vaccine protects mice against *Escherichia coli* heat-labile enterotoxin (LT): potatoes expressing a synthetic *LT-B* gene. Vaccine 1998;16:1335–42.

[11] McGarvey PB, Hammond J, Dienelt M, Hooper DC, Fu ZF, Dietzschold B, et al. Expression of rabies virus glycoprotein in transgenic tomatoes. Biotechnology 1995;13:1484–7.

[12] Wigdorovitz A, Carrillo C, Dus Santos MJ, Sadir AM, Ríos R, Franzione P, et al. Induction of a protective antibody response to foot and mouth disease virus in mice following oral or parenteral immunized with alfalfa transgenic plants expressing the viral structural protein VP1. Virology 1999;255:347–53.

[13] Brown F. Vaccination against foot and mouth disease virus. Vaccine 1992;10:1022–6.

[14] Carrillo C, Wigdorovitz A, Trono K, Dus Santos MJ, Castañón S, Sadir AM, et al. Induction of a virus-specific antibody response to foot and mouth disease virus using the structural protein VP1 expressed in transgenic potato plants. Viral Immunol 2001;14:49–57.

[15] Zamorano P, Wigdorovitz A, Pérez-Filgueira M, Carrillo C, Escribano JM, Sadir AM, et al. A 10 amino acid linear sequence of VP1 of foot and mouth disease virus (FMDV) containing B and T cell epitopes induces protection in mice. Virology 1995;212:614–21.

[16] Wen-Jun S, Forde BG. Efficient transformation of *Agrobacterium* spp. by high voltage electroporation. Nucl Acid Res 1989;17:8385.

[17] Shetty K, McKersie BD. Proline, thioproline and potassium-mediated stimulation of somatic embryogenesis in alfalfa (*Medicago sativa* L.). Plant Sci 1993;88:185–93.

[18] McKersie BD, Chen Y, de Beus M, Bowley SR. Superoxide dismutase enhances tolerance of freezing stress in transgenic alfalfa (*Medicago sativa* L.). Plant Physiol 1993;103:1155–63.

[19] Klimyuk VI, Carroll BJ, Thomas CM, Jones JDG. Alkali treatment for rapid preparation of plant material for reliable PCR analysis. Plant J 1993;3:493–4.

[20] Gallagher SR. The GUS assay. In: Gallagher SR, editor. GUS protocols: using *GUS* gene as a reporter gene expression, 1992.

[21] Berinstein A, Piatti P, Gaggino OP, Schudel AA, Sadir AM. Enhancement of the immune response elicited with foot and mouth disease virus vaccines by an extract of the *Mycobacterium* sp. wall. Vaccine 1991;9:883–8.