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Induction of a Protective Antibody Response to Foot and Mouth Disease Virus in Mice Following Oral or Parenteral Immunization with Alfalfa Transgenic Plants Expressing the Viral Structural Protein VP1[1]

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The utilization of transgenic plants expressing recombinant antigens to be used in the formulation of experimental immunogens has been recently communicated. We report here the development of transgenic plants of alfalfa expressing the structural protein VP1 of foot and mouth disease virus (FMDV). The presence of the transgenes in the plants was confirmed by PCR and their specific transcription was demonstrated by RT-PCR. Mice parenterally immunized using leaf extracts or receiving in their diet freshly harvested leaves from the transgenic plants developed a virus-specific immune response. Animals immunized by either method elicited a specific antibody response to a synthetic peptide representing amino acid residues 135–160 of VP1, to the structural protein VP1, and to intact FMDV particles. Additionally, the immunized mice were protected against experimental challenge with the virus. We believe this is the first report demonstrating the induction of a protective systemic antibody response in animals fed transgenic plants expressing a viral antigen. These results support the feasibility of producing edible vaccines in transgenic forage plants, such as alfalfa, commonly used in the diet of domestic animals even for those antigens for which a systemic immune response is required.

Key Words: FMDV; infectious immunity virus; vaccination.

INTRODUCTION

The utilization of plants for the production of foreign proteins to be used as experimental immunogens was first reported by Mason *et al.* in 1992. Since then, several groups have used transgenic plants for expressing a variety of viral and bacterial antigens (Arakawa *et al.,* 1998; Carrillo *et al.,* 1998; Gómez *et al.,* 1998; Haq *et al.,* 1995; Mason *et al.,* 1996; McGarvey *et al.,* 1995). Plants producing recombinant foreign proteins could be an inexpensive source of antigens that could be easily purified for parenteral inoculation. However, only a reduced number of viral or bacterial antigens produced in plants have been tested for their immunogenicity when orally administered. Some examples are the Norwalk virus capsid (Mason *et al.,* 1996), the *Escherichia coli* heat labile enterotoxin (Haq *et al.,* 1995), and the cholera toxin B subunit (Arakawa *et al.,* 1998). In addition to the possibility of using plants as bioreactors for the production of vaccine antigens, as an alternative to conventional fermentation-based procedures, their utilization as a system for delivering antigens by the oral route, by including them in the diet, has pointed out the necessity of studying the oral immunogenicity of a wide variety of antigens expressed by this methodology.

Foot and mouth disease virus (FMDV) is the causative agent of an economically important disease affecting meat-producing animals (Brown, 1992). Comprehensive vaccination of all susceptible hosts, using inactivated virus as immunogen, constitutes the basis of all sanitary plans for the control and eradication of the disease (Brown, 1992). The structural protein VP1 carries critical epitopes responsible for the induction of neutralizing antibodies. The expression of immunogenic areas of VP1 in a diverse range of prokaryotic and eukaryotic systems has been performed, resulting in effective experimental immunogens (DiMarchi *et al.,* 1986; Kleid *et al.,* 1981; Morgan and Moore, 1989; Zamorano *et al.,* 1995).

In the present study we utilized VP1 of FMDV serotype O1 Campos (O1C) as a model to investigate the possibility of using transgenic alfalfa plants as a source of antigen in the production of a recombinant immunogen as well as an experimental edible vaccine. The results presented in this report demonstrate that mice, parenterally or orally immunized with leaves obtained from transgenic plants, developed a similar virus-specific im-

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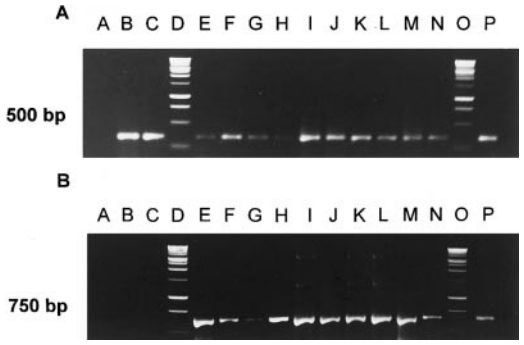

FIG. 1. Detection of the VP1 and *nptII* genes in transgenic plants using PCR. Plant DNA was isolated from cell extracts and PCR was performed with a pair of primers that specifically amplify DNA fragments of 344 bp from the *nptII* gene (A) and 655 bp from the VP1 gene (B), respectively. Lane description: nontemplate (A), DNA from pRok (kanamycin-resistant individuals) transformed plants (B and C), DNA from pRok.VP1a transformed plant (E–N), pRok.VP1a plasmid (P), MW marker (D and O).

mune response that was able to protect the animals from experimental challenge with the virulent virus.

RESULTS

Production and genetic analysis of transformed plants

Recombinant pRok.VP1a plasmids (pRok2 binary vector using the cauliflower mosaic virus, CaMV, 35S promoter to drive nominally constitutive transcription of the cloned genes) also contain the selectable marker *nptII,* which permits selection of transformed plants on medium containing kanamycin. Both genes, VP1 and *nptII,* are located in the TDNA region of the plasmid, allowing the stable integration of the recombinant DNA into the nuclear chromosomal DNA of the plant. Plant transformation with pRok.VP1a was mediated by *Agrobacterium tumefaciens.*

The presence of the VP1 gene in the transgenic plants was detected by PCR. The analysis showed the presence of an amplified product of the expected size (655 bp) in all plants transformed with pRok.VP1a (Fig. 1). This product was absent in nontransformed plants as well as in kanamycin-resistant plants harboring an unrelated gene. As control, the presence of the *nptII* gene was also investigated in the same DNA plant samples. A specific amplification product of 344 bp was consistently present in all kanamycin-resistant plants but absent from the nontransformed individuals (Fig. 1).

Detection of transcriptional activity in the transgenic plants

The analysis of transcription of specific genes in the transformants was performed by RT-PCR. Plants transformed with pRok.VP1a presented active transcriptional activity corresponding to the recombinant genes of VP1 and *nptII* along with the housekeeping ITS gene fragment, whereas the nontransformed plants showed the transcription of only the ITS gene fragment (Fig. 2). To rule out the possibility of amplification of contaminant DNA in the samples, direct PCR amplification without reverse transcription was performed on the RNA preparations. No amplified DNA fragments were detectable under those conditions, confirming the RNA specificity of the reaction (Fig. 2).

Selection of transgenic plants expressing VP1 and induction of immune response in intraperitoneally immunized mice

The presence of the recombinant protein in all the developed alfalfa clones harboring the VP1 gene was tested by direct ELISA using the anti-FMDV serum. Ten of 15 lines were positive in at least three sequential determinations (data not shown) and were then selected for the immunization experiments.

Balb/c mice were immunized ip at days 0, 15, 30, and 45 with approximately 15 to 20 mg of plant tissue emulsified in incomplete Freund's adjuvant (plant extracts were prepared by macerating approximately 50 mg of frozen leaves in 1 ml of PBST). Ten days after the last inoculation, animals were bled and the sera analyzed for the presence of anti-FMDV antibodies. The experiment was independently performed twice (Groups A and B in Fig. 3).

Antibodies raised in immunized mice showed a strong response in ELISA against VP1 as demonstrated by their reactivity to p135-160 (Figs. 3A and 3B). The specificity of this anti-VP1 response was confirmed by Western blot, using purified FMDV as anti-

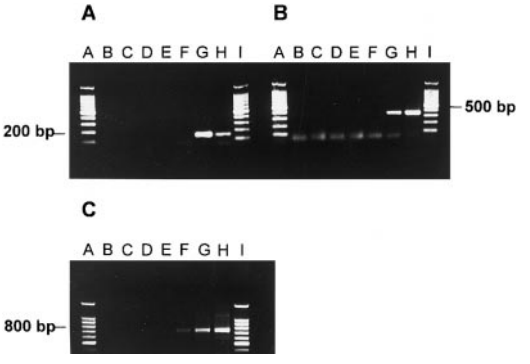

FIG. 2. Detection of transcription of the VP1, *nptII,* and ITS genes in transgenic plants using RT-PCR. Plant RNA was isolated from cell extracts and RT-PCR was performed with a pair of primers that specifically amplified a DNA fragment of 145 bp of the VP1 gene (A), 344 bp of the *nptII* gene (B), and approximately 800 bp containing the ITS fragment (C), respectively. Lane description: RNA samples shown in lanes B, C, and D were subjected to PCR amplification without the previous RT reaction as control for DNA contamination. RNA samples shown in lanes E, F, and G were subjected to PCR amplification after RT reaction. Nontemplate RT (B and E), RNA from nontransformed plants (C and F), RNA from pRok.VP1a transformed plants (D and G), DNA for the control of amplification (H), MW marker (A and I).

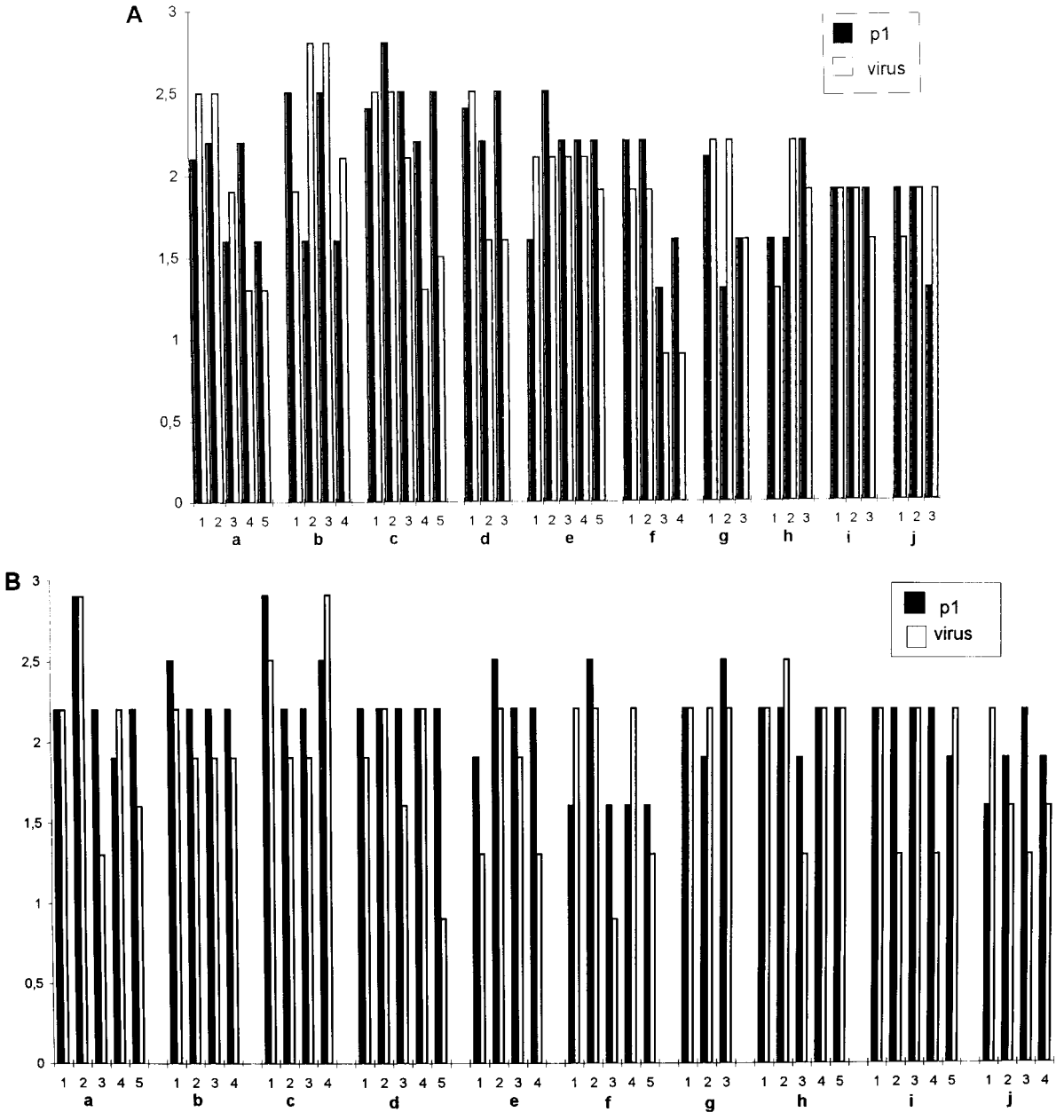

FIG. 3. Detection of antibodies to p135-160 (p1) (solid bars) and anti-FMDV particles (virus) (open bars) in mice intraperitoneally immunized with leaf extracts from transformed plants, using ELISA. The figure shows the results of two independently performed experiments (A) and (B). Numbers correspond to the mice immunized with each of the 10 different pRok.VP1a transformed plants (a–j). Serum titers are expressed as the $\log_{10}$ of the reciprocal of the highest serum dilution that presents OD readings above of the mean OD readings + 3 SD of sera from five animals immunized with nontransformed plants.

gen; a pool of sera from mice immunized with plants expressing the recombinant protein specifically recognized a protein with the same relative mobility as that recognized by an immune serum raised against p135-160 (Fig. 4). Additionally, the specific immune response against FMDV intact particles was analyzed by ELISA. All animals immunized with plants expressing VP1 developed a strong immune response (Figs. 3A and 3B). Sera from mice immunized with leaf extract from nontransformed plants showed no reactivity in either ELISA or Western blot, supporting the specificity of the immune response induced by the extracts from plants containing VP1 (Figs. 3A, 3B, and 4).

Induction of an immune response in orally immunized mice

For the oral immunization experiments adult male Balb/c mice were fed with approximately 0.3 g of freshly harvested leaves each time, three times a week for 2

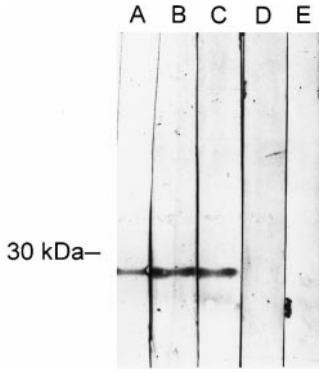

FIG. 4. Anti-VP1 antibodies detected by Western blot in the sera from mouse immunized either orally or ip with leaves from recombinant plants. Lane description: a pool of sera from mice fed (A) or ip immunized (B) with leaves from pRok.VP1a transformed plants, an anti-p135-160 serum (C), and a pool of sera from mice fed (D) or ip immunized (E) with leaves from nontransformed plants, respectively.

months. One of the groups received leaves from a pool of the 10 plants expressing VP1 utilized in parenteral immunization, while the control group was fed leaves obtained from nontransformed plants. Ten days after the last feeding the presence of anti-FMDV antibodies was checked in the blood of the immunized mice. The experiment was performed twice (Groups A and B in Fig. 5), independently. The orally immunized mice showed a specific reaction in ELISA against VP1, as determined by their reactivity against p135-160 (Figs. 5A and 5B). Again, this specificity was confirmed by Western blot analysis using purified FMDV as antigen; a pool of the orally immunized mice recognized a protein of a size similar to that recognized by the anti-p135-160 serum. Finally, all orally immunized mice developed a specific antibody response against whole virus particle (Figs. 5A and 5B). Sera from mice fed leaves from nontransformed plants showed no reactivity in any of the tests performed (Figs. 4, 5A, and 5B).

Immunized animals were protected against the virus challenge

To test the effectiveness of the induced immune response in preventing infection following virus exposure, the groups of mice orally or intraperitoneally immunized with leaves from alfalfa plants expressing VP1 (Figs. 3A, 3B, 5A, and 5B) were experimentally challenged with FMDV. Mice were inoculated ip with 10^4 suckling mouse lethal doses ($SM_{50}LD$) of FMDV O1C and 36 h later the absence of viremia was considered an indicator of protection. Interestingly, 77 to 80% of the animals immunized intraperitoneally were protected. More importantly, 66 to 75% of the animals fed leaves from VP1 expressing plants showed protection after the experimental challenge (Tables 1 and 2).

DISCUSSION

Since the concept of using transgenic plants for vaccine production was first described by Mason *et al.* (1992) several authors have described the expression of vaccine antigens using this methodology (Arakawa *et al.,* 1998; Carrillo *et al.,* 1998; Gómez *et al.,* 1998; Haq *et al.,* 1995; Mason *et al.,* 1996; McGarvey *et al.,* 1995). The demonstration that some of these antigens were immunogenic when orally administered (Arakawa *et al.,* 1998; Haq *et al.,* 1995; Mason *et al.,* 1996) encouraged the study of other antigens expressed in plants in order to develop edible vaccines. We have previously demonstrated that protein VP1 could be expressed as an immunogenic antigen in *Arabidopsis,* an experimental plant system (Carrilo *et al.,* 1998). Now, we have extended our studies by expressing this protein in alfalfa, a forage

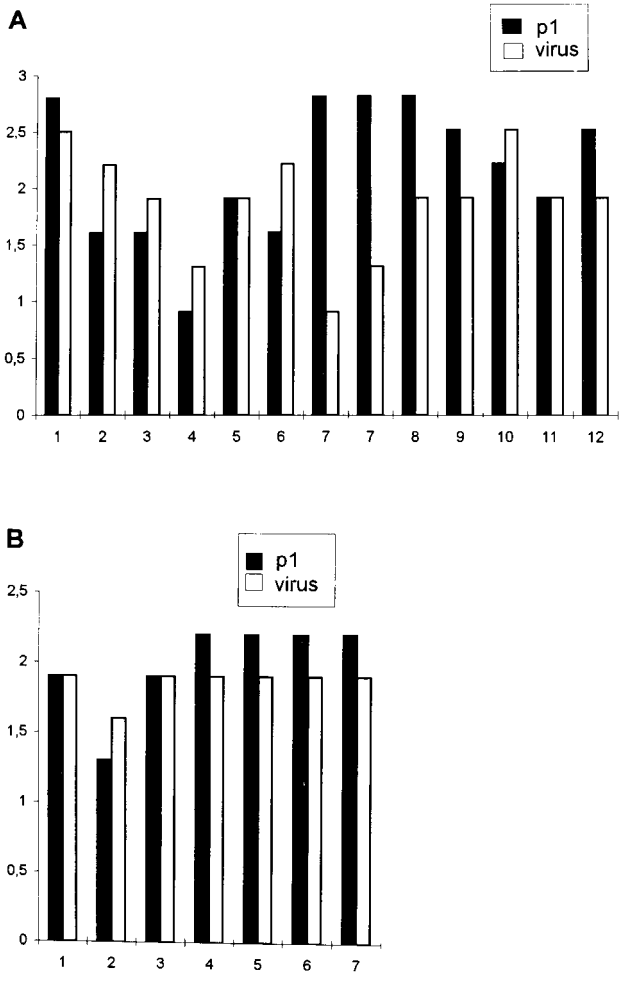

FIG. 5. Detection of antibodies to p135-160 (p1) (solid bars) and anti-FMDV particles (virus) (open bars) in mice fed freshly harvested leaves from transformed plants, using ELISA. The figure shows the results from two independently performed experiments (A) and (B). Numbers correspond to the mice fed leaves from a pool of the 10 different pRok.VP1a transformed plants used in Fig. 3. Serum titers are expressed as in Fig. 3.

TABLE 1

Protection against FMDV Challenge in Mice ip Immunized with Plant Extracts

Mice immunized ip with	Protection rate[a] Experiment A	Experiment B
pRok.VP1a plant extracts	10/13 (77%)	16/20 (80%)
Nontransformed plant extracts	0/6 (0%)	0/6 (0%)
Mock immunized	0/6 (0%)	0/6 (0%)

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

plant, which can be produced in enough quantity to feed domestic species susceptible to FMDV. In the present work we described the production of transgenic plants expressing the structural protein VP1 of FMDV and their utilization as a source of antigen for the immunization of experimental hosts. The plant-derived VP1 was able to induce, in parenterally immunized mice and, more importantly, in mice fed leaves from the transgenic plants, a virus-specific antibody response and protection against virulent challenge.

All the plant-expressed antigens, which were reported to be immunogenic when orally administered, were proteins belonging to microorganisms whose native habitat is the enteric tract (Arakawa *et al.,* 1998; Haq *et al.,* 1995; Mason *et al.,* 1996). Thus, during the immunization process, it is expected that these antigens will show a certain natural resistance to the physiological digestive mechanisms with the concomitant maintenance of their immunogenicity. Interestingly, we found that mice fed fresh leaves expressing VP1 presented a systemic immune response to FMDV that was protective against the experimental challenge performed by the intraperitoneal route. We believe this is the first report showing a systemic protective immune response induced by oral immunization with a viral antigen expressed in transgenic plants. In fact, this result is in agreement with our data that demonstrated that mice orally immunized with purified FMDV particles (as well as with OVA, as a control antigen) developed a significant antigen-specific systemic antibody response and protection against experimental challenge (Peralta *et al.,* unpublished results).

Although its quantification was not performed, the level of expression of VP1 in the analyzed transgenic plants was not very significant. The difficulty in detecting the foreign protein in the plant extract, by Western blot, and the necessity of performing several immunizations in order to induce a significative immune response indicate the scarcity of the expressed protein. Thus, the increase of the amount of foreign protein in the transgenic plants should be a goal in the near future. The use of alternative promoters and, principally, the adaptation of the codon usage in the foreign genes to that used in plants could improve expression levels.

In summary, the results presented here support the concept of using transgenic plants as a novel and safe system for vaccine production, which could become a very attractive alternative in the developing world. The fact that feeding mice transgenic plants expressing a recombinant antigen caused an oral immune response demonstrates the feasibility of using transgenic plants as expression and delivery systems for oral vaccines, even against those antigens for which a systemic immune response is required. The establishment of food plant-based oral immunization method for microbial antigens could be an inexpensive alternative to conventional fermentation systems for vaccine production, as food plants can be grown inexpensively in large quantities and are easily administered.

MATERIALS AND METHODS

Production of transgenic plants of alfalfa

A 655-bp DNA fragment encoding the VP1 gene was amplified by RT-PCR from viral RNA using specific primers (forward primer, 5′ AGCGGATCCTGTCATGGCCACTGTTGAA 3′; reverse primer, 5′ AAGGGGATCCTCTAGAGTCTACTTCAG 3′) and cloned into the binary pRok2 plasmid (Baulcombe *et al.,* 1986) under the control of the cauliflower mosaic virus (CaMV 35S) promoter (recombinant plasmid pRok.VP1a). The designed recombinant product starts at codon 13 of the native VP1 gene and contains the complete gene sequence including its native carboxy end. The recombinant binary vector was introduced in *A. tumefaciens* strain LBA 4404 by electroporation using the procedure described by Wen-Jun and Forde (1989). Petioles of alfalfa clone C2-3, kindly provided by Drs. B. McKersie and S. Bowley (Plant Biotechnology Division, Department of Plant Agriculture, University of Guelph, Guelph, Ontario, Canada) were cocultivated with *A. tumefaciens* and cultured *in vitro* as described by Shetty and McKersie (1993). The *in vitro* selection was performed using 50 mg/ml of kanamycin as described by McKersie *et al.* (1993).

TABLE 2

Protection against FMDV Challenge in Mice Fed Plant Leaves

Mice fed with	Protection rate[a] Experiment A	Experiment B
pRok.VP1a plant leaves	6/9 (66%)	6/8 (75%)
Nontransformed plant leaves	0/5 (0%)	0/6 (0%)
Mock immunized	0/5 (0%)	0/6 (0%)

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

PCR and RT-PCR analyses

The presence of the recombinant genes in transgenic alfalfa plants was detected by PCR (Carrillo *et al.,* 1998). Total nucleic acids were extracted from samples of approximately 50 mg of leaves following the protocol described by Klimyuk *et al.* (1993). The VP1 gene was amplified using a pair of primers that span from position 63 of VP1 (forward primer, 5′ AGCGGATCCTGTCATGGCCACTGTTGAA 3′) to position 27 of p52 (reverse primer, 5′ AAGGGGATCCTCTAGAGTCTACTTGAG 3′). The presence of the *nptII* gene, which confers resistance to kanamycin, was detected by PCR using a pair of specific primers (forward primer, 5′ CAGACAATCGGCTGCTCTGAT 3′; reverse primer, 5′ TGCGATGTTTCGCTTGGTGT 3′) that amplify an internal fragment of 344 bp (Desganés *et al.,* 1995). PCRs were performed in a 50-μl final reaction volume, containing 5 μl of plant DNA, 2.5 mM $MgCl_2$, 100 μM of dNTPs, and 0.5 μM of each primer.

The transcription of the transgenes was analyzed by specific amplification of VP1 transcripts from plant extracts. Total RNA extraction was performed from 1 g of fresh alfalfa leaves, which were ground in the presence of liquid nitrogen and resuspended in 2 ml of TE and 8 ml of Trizol (Gibco BRL). After chloroform extraction (0.2 ml of chloroform per milliliter of plant suspension) the aqueous phase was precipitated with 0.5 ml of isopropanol per milliliter of plant extract. Pellets were washed with 70% ethanol, dried, and resuspended in 200 μl of TE. DNA was digested with RNase-free DNase (Boehringer). The absence of residual DNA in the RNAs obtained was checked by PCR. RT reaction was carried out in a 50-μl final reaction volume that contained 2 mM random priming hexamers and a mixture of dNTPs (at a concentration of 1 mM/each), 1 unit of RNase inhibitor, and 10 units of MMLV-RT enzyme in 1× RT buffer. The mixture was incubated for 30 min at 37°C and then inactivated for 10 min at 95°C. Samples were cooled and held at −20°C until used for PCR amplification. PCR was performed using primers that specifically amplify a 145-bp fragment of the VP1 gene between positions 369 and 490 (forward primer, 5′ CCGGATCCTGGCAACATGTACA 3′; reverse primer, 5′ GGTCGCATATGTGGCACCGTAGTT 3′). As positive control for RNA extraction, a fragment of approximately 800 bp that encompasses the 5.8S mRNA gene and the flanking intragenic transcribed spacers (ITS) was amplified using specific primers (forward primer, 5′ GGAAGGAGAAGTCGTAACAAGG 3′; reverse primers, 5′ TCCTCCGCTTATTGATATGC 3′). The same *nptII* primers described above for the PCR technique were used as an additional control for RNA detection.

Analysis of foreign protein expression in transgenic plants

The screening of the recombinant plants was performed by ELISA. Ninety-six-well Immulon 2 ELISA plates (Dynatech) were coated with a rabbit anti-FMDV antiserum in carbonate buffer, pH 9.6, for 12 h at 4°C. Plates were then washed three times with PBS containing 0.05% Tween 20 (PBST) and blocked with 3% horse serum in PBST for 2 h at 37°C (all subsequent steps were performed using this buffer). A four fold dilution of leaf extracts from the plants to be tested was then added and incubated for 1 h at 37°C. Plates were washed and a pool of mouse anti-FMDV antiserum was added and incubated for 1 h at 37°C. Plates were washed again and incubated for 45 min at 37°C with peroxidase-labeled rabbit anti-mouse Ig (Dakkopats). After five washes, the reaction was developed by addition of *o*-phenylenediamine–H_2O_2 in citrate buffer, pH 5, and read 3 min later at 490 nm in an MR 500 Microplate Reader (Dynatech). Plants presenting consistently (in at least three consecutive independent determinations) OD readings of 3 standard deviations (SD) over the nontransgenic plant used as control were selected for further studies.

Analysis of antibody response to plant-expressed VP1 polypeptides

Adult (60 to 90 days old) male BALB/c mice were immunized intraperitoneally on days 0, 15, 30, and 45 with either leaf extracts from transgenic plants expressing VP1 or leaf extracts from nontransformed plants of alfalfa (150 μl of leaf extract, containing 15 to 20 mg of fresh leaf tissue, in incomplete Freund's adjuvant per animal per injection). Orally immunized mice were fed three times a week, for 2 months with approximately 0.3 g of freshly harvested leaves each time.

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

ELISA. ELISA was performed using as antigen either a synthetic peptide (p135-160), which represents the amino acid residues of FMDV VP1 O1C between positions 135 and 160 (Zamorano *et al.,* 1995), or complete FMDV particles. The assay was performed as described for detection of VP1 in transgenic plants for the capture of antigen to the plate, blocking, washing, developing, and reading steps (Pérez Filgueira *et al.,* 1995). In the case of p135-160, peptide was directly adsorbed to the plate at a concentration of 15 μg/ml. To detect anti-virus particle activity, purified FMDV particles (produced, inactivated, and purified as described in Berinstein *et al.,* 1991) were added to the plate (at a concentration of 1 μg/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 that gives OD readings above the mean OD + 3 SD of sera from five animals immunized with nontransformed plants.

Western blot. Inactivated purified FMDV was resuspended in sample buffer (50 mM Tris, pH 7.5, 1 mM

PMSF, 8 M urea, 1% SDS, 2 mM DTT, and 2% 2-βME), boiled for 10 min, electrophoresed in 12.5% SDS–PAGE, and blotted to an Immobilon P (Millipore) membrane. The membrane was blocked overnight with PBST containing 3% skim milk (all subsequent steps were performed using this buffer) and incubated with the corresponding mouse sera (diluted 1/20) for 2 h at 37°C. The membrane was then washed using PBST and incubated with an alkaline phosphatase labeled anti-mouse Ig rabbit antiserum (Dakkopats) for 1 h at 37°C. After being washed three times the reaction was developed by the addition of the substrate NBT/CIP.

Challenge experiments

Mice were challenged ip with 10^4 $SM_{50}LD$. Protection was determined by absence of viremia in the challenged mice at 36 h postinfection. Viremia was tested by im inoculation of 50 μl of a 1/10 dilution of peripheral blood to a 5- to 6-day-old litter of six mice per blood sample.

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