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Special Feature

Transgenic plants for the production of veterinary vaccines

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Summary The expression of antigens in transgenic plants has been increasingly used in the development of experimental vaccines, particularly oriented to the development of edible vaccines. Hence, this technology becomes highly suitable to express immunogenic proteins from pathogens. Foot and mouth disease virus, bovine rotavirus and bovine viral diarrhoea virus are considered to be the most important causative agents of economic loss of cattle production in Argentina, and they are thus optimal candidates for alternative means of immunization. Here, we present a review of our results corresponding to the expression of immunogenic proteins from these three viruses in alfalfa transgenic plants, and we discuss the possibility of using them for the development of plant-based vaccines.

Key words: antigen expression, bovine rotavirus (BRV), bovine viral diarrhoea virus (BVDV), foot and mouth disease virus (FMDV), recombinant plant.

Introduction

The use of transgenic plants for the production of viral and bacterial antigens has been frequently reported in past years. This biotechnological strategy has the enormous advantage of not requiring fermentation control systems under strict conditions of biosafety and sterility, which makes this technology particularly attractive for its simplicity and low cost.

In addition to the utilization of plants as bioreactors for the production of vaccine antigens, plants also provide an adequate system for oral delivery of recombinant immunogens by including them in the diet. A number of viral and bacterial antigens produced in plants have already demonstrated their immunogenicity when orally administered.[1–7] This approach is particularly relevant for enteric pathogens, because oral immunization may be able to elicit appropriate immune mechanisms for the induction of protective responses. In all of these studies, the concentration of the expressed protein in the transgenic plant tissues was relatively poor.

Foot and mouth disease virus (FMDV), bovine rotavirus (BRV) and bovine viral diarrhoea virus (BVDV) are considered to be the most important causative agents of economic loss in Argentinean cattle production, so they are optimal candidates for finding alternative means of immunization against them. In this paper, we present the development of different experimental immunogens of these viruses through the expression of immunogenic proteins in alfalfa transgenic plants.

Foot and mouth disease virus

Foot and mouth disease virus is the causative agent of a very significant economic disease affecting meat and milk producing domestic animals.

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The disease is present in many regions of the world, although it has been successfully eliminated from Europe and Central and North America. Argentina has not had an outbreak since 2003, and it has recovered its status as a foot and mouth disease (FMD)-free country with vaccination, in January 2005. Nevertheless, there is still risk of introduction of virus to the country due to its proximity to areas where the disease is endemic.

Infection with FMDV induces a cellular and humoral immune response in hosts. The production of neutralizing antibodies is responsible for conferring protection to viral infection.

Current FMDV vaccines use inactivated virus and, although they have proved to be effective tools for the prevention of the disease, their production is both costly and risky because manipulation of massive amounts of virulent virus could result in virus dissemination.[8] Thus, it is important to develop practical alternatives to the current methodology of vaccine production.

With that in mind, we have focused on the development of alternative methods of FMDV vaccine production using transgenic plants as bioreactors. First, we used the structural protein VP1, which carries critical epitopes responsible for the induction of protective neutralizing antibodies, as a model to investigate the possibility of using transgenic plants as a source of antigen. We have successfully demonstrated that VP1 can be expressed in *Arabidopsis thaliana*, alfalfa and potato plants,[5,9,10] and showed that those materials elicited an antibody response and protection against virulent challenge when parenterally or orally administered as experimental immunogen.

Nevertheless, in all those cases,[5,9,10] the concentration of the expressed protein in the transgenic plant tissues was relatively low. The difficulty in detecting the foreign protein in the plant extract as well as the necessity of numerous immunizations to induce a significant immune response indicate the low level of the expressed protein. This point becomes particularly relevant in those cases where the plant extracts are expected to be used without any further processing.

Consequently, increasing the concentration of the foreign proteins in transgenic plants constitutes a critical issue to be considered.

Among other strategies, including genetic manipulation, a way to solve this problem could be identification of those transgenic events expressing exceptionally high levels of the recombinant protein. For this purpose, it becomes 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.

We have developed a methodology based on 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 peptide of choice to be evaluated was the area comprised by amino acid residues 135–160 from the structural protein VP1 of FMDV (p135–160). The results showed that a large number of individuals can be readily screened by their β glucuronidase (βGUS) enzymatic activity.[11] Mice immunized using the selected plants developed a strong and protective antibody response against virulent FMDV in experimental hosts.

Although plant expression is a very promising technology, most of the antigens expressed to date have been epitopes or single proteins containing linear epitopes, or simple 3-D structures.[3,7,9–14] However, the practical use of transgenic plants as a source of antigen for vaccine production would undoubtedly require, in most cases, the expression of more complex antigenic structures. In the case of FMDV, an alternative approach to conventional vaccines is the preparation of empty capsids by expression and processing of the precursor in a heterologous expression system. Empty capsids maintain continuous and discontinuous B-cell epitopes presented in authentic virion as well as T-cell epitopes identified in cattle and swine.[15] In addition, it has previously been shown that empty FMDV capsids are capable of eliciting the same antibody response as infectious FMDV particles.[16]

Based on these results, our approach toward the development of plant-based vaccines has been to express the capsid coding region P1-2A and the protease 3C coding region, necessary for processing P1 to the four capsid proteins in alfalfa plants. The expressed products were able to evoke an immune response in a mouse experimental model in terms of inducing a strong FMDV-specific antibody response against complete virus particles and viral subunits, as well as complete protection against experimental challenge with virulent virus.

Bovine rotavirus

Rotaviruses are the principal aetiological agents of severe acute gastroenteritis in numerous mammalian species throughout the world.[17] Newborn calves are susceptible to rotavirus infection during the first weeks of life, making it difficult to actively immunize the animals before exposure to the virulent pathogen. However, colostral antibodies produced in rotavirus-vaccinated mothers were reported to confer passive protection to the newborn, as shown in an experimental mouse model[18] as well as in the natural host.[19–21]

Bovine rotavirus epitope *eBRV4* has been described as harbouring at least one neutralizing epitope as well as being responsible for the adsorption of virus to epithelial cells. We produced transgenic alfalfa plants expressing the chimeric protein *eBRV4*-βGUS. The plants were produced and efficiently selected based on their levels of βGUS enzymatic activity. The *eBRV4* epitope expressed in plants was effective in conferring passive protection as it was evaluated using a suckling mouse model.

Bovine viral diarrhoea virus

Bovine viral diarrhoea virus is the causal agent of a worldwide disease that infects bovines of all ages, causing reproduction problems and altering biological products of high commercial value, resulting in considerable industrial losses. Efficacy of commercially available inactivated vaccines is a controversial issue due to the diversity of circulating strains, and the absence of experimental evidence of protection levels during different outcomes of the infection.[22–25] Therefore, the introduction of more efficacious vaccines is demanded by veterinarians and farmers. The BVDV genome encodes at least 10 structural proteins. One of them, E2, contains the main antigenic sites for the production of neutralizing antibodies that play a predominant role in the defence against infection.[26–28] Immunization with E2 has been demonstrated to induce neutralizing antibodies in experimental and natural hosts.[29,30]

This glycoprotein, then, is an excellent immunogen in experimental subunit vaccines. Unfortunately, none of the eukaryotic expression systems currently available allows the industrial production of recombinant glycoprotein at a low cost.

For the expression of BVDV glycoprotein E2 in alfalfa, the genetic construction was optimized by the incorporation of two different sequences that have been previously used as efficient potentiators of the expression level of foreign proteins in transgenic plants: (i) the TEV virus leader sequence[31,32] and (ii) the microsome retention sequence SEKDEL.[33]

Here, we present a review of our results corresponding to the expression of immunogenic proteins from these three viruses in alfalfa transgenic plants, and we discuss the possibility of using them for the development of plant-based vaccines.

Results

Foot and mouth disease virus

VP1 expression in A. thaliana*, potato and alfalfa*

The structural protein VP1 carries critical epitopes responsible for the induction of neutralizing antibodies. The expression of immunogenic areas of VP1 in diverse prokaryotic and eukaryotic systems has been performed, resulting in effective experimental immunogens.[34–37]

We used VP1 of FMDV serotype O1 Campos (O1C) as a model to investigate the possibility of using transgenic plants as a source of antigen in the production of a recombinant immunogen as well as an experimental edible vaccine. With that purpose in mind, we expressed VP1 in *A. thaliana*, potato and alfalfa.

The vector used to deliver the transgenes in the plant genome is a binary plasmid (pROK-VP1) that contains the

Cauliflower Mosaic Virus (CaMV35S) promoter to direct constitutive expression of the foreign genes. The genetic transformation of plants was conducted by the *Agrobacterium tumefaciens* methodology.

VP1 expression was, in all cases, evaluated by ELISA. The recombinant protein could be detected in 10% of *A. thaliana*, 65% of potato and 60% of alfalfa plants. These plants were used for parenteral immunization assays; additionally, fresh leaves from selected alfalfa plants were used to feed mice. Both parenteral and oral immunization induced a strong specific antibody response.[5,9,10] To test the effectiveness of the induced immune response in preventing infection following virus exposure, groups of mice orally or intraperitoneally immunized were experimentally challenged with FMDV. All immunized mice were protected against viral infection.

Optimization of expression levels

Increasing the amount of antigen expressed in transgenic plants is a critical issue. We developed a methodology that allows easy and rapid selection of plants showing the highest expression levels. We generated fusion proteins with an immunorelevant peptide and a reporter gene. The FMDV peptide used was the area between residues 135 and 160 of FMDV VP1. The native p135–160 DNA sequence was adapted to the more frequent *A. thaliana* plant codon usage.[38] The p135–160 DNA fragment was cloned in the plasmid pBI121 (Clontech, Palo Alto, CA, USA), which contains the *gus* A gene under the control of the CaMV35S promoter. The plasmid (pBI121p135–160-βGUS) encodes 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. This construction was used to obtain transgenic alfalfa plants. The screening of plants was evaluated by their βGUS activity; the individuals assayed showed a high degree of βGUS activity, from basal values to highest levels (8% of individuals) (Fig. 1A).

Those plants presenting the highest level of βGUS activity (plant numbers 5, 11, 12 and 29) were selected for further analyses of the expression of p135–160-βGUS fusion protein.

It was observed that, coincidentally with the degree of βGUS activity in each of the tested plants, the individuals having the highest βGUS expression (plants 5 and 29) also had the highest levels of p135–160 peptide accumulation (Fig. 1B). The approximate concentration of the recombinant p135–160-βGUS expressed in the transgenic line 5 was estimated to range between 0.5 and 1 mg/g of total soluble protein (TSP).

With the purpose of studying the stability of the expressed product, the expression of the fusion protein was analysed during the lifetime as well as during the asexual reproduction cycles. The expression level of plant number 5 remained practically unchanged when monitored over the 26 month experimental period. In addition, no significant variation was observed in the expression levels of p135–160-βGUS in a study carried out on 14 individuals obtained by vegetative reproduction of plant number 5.

The immunogenicity of the p135–160 epitopes expressed in the recombinant plants was tested by immunizing mice with crude extracts from plant number 5. After three doses, all of the sera from the p135–160-βGUS immunized mice presented a specific antibody response against FMDV antigens (Fig. 2A,B). When these animals were challenged with virulent virus, they were completely protected against infection (Table 1).

Expression of complex antigenic structures

Empty capsids of FMDV are an optimal antigen to generate a vaccine in terms of security and efficacy as an immunogen.

The genetic construction used involved the precursor P1.2A (from which the four viral structural proteins are generated), a fragment of 2B, 3B and the protease 3C (P1-3C).

It is reported that the expression of P1.2 A together with 3C resulted in the production of FMDV empty capsids carrying the correct conformation.[39,40] The DNA fragment containing the coding sequences for the production of empty capsids was cloned in the binary vector pROK. In this plasmid, transcription of transgenes is under the control of the CaMV35S promoter. Transgenic alfalfa plants were obtained by the *A. tumefaciens* methodology. By reverse transcription (RT)–PCR criteria, pROKP1-3C-transformed plants consistently presented active transcription of the recombinant P1-3C genes.

These plants (presenting an expression level of 0.01–0.2 mg/g TSP) were used to immunize mice. After four doses, the antibodies raised in the immunized mice presented a specific response in ELISA against the synthetic peptide p135–160, and to purified FMDV particles.

Based on these results, plant A was chosen to further continue with the immunological analysis of the product expressed in alfalfa tissues. The antibody response induced by the vaccine formulated with leaves of plant A was confirmed by immunizing a new group of mice and analysing their sera by ELISA (Fig. 2C). The specificity in the antibody response was confirmed by western blot, using purified FMDV as antigen. Different pools of sera from the immunized mice specifically recognized the FMDV structural proteins with a similar pattern of reactivity as a pool of sera from mice experimentally infected with FMDV (Fig. 2D).

Furthermore, mice immunized with plant A extract developed a strong neutralizing antibody response (Fig. 2E). To test the effectiveness of the induced immune response in preventing infection following virus exposure, the vaccinated mice were experimentally challenged with infectious FMDV. All immunized animals were protected against the experimental infection, indicating the capability of the plant expressing FMDV polyprotein P1-3C in inducing a protective antibody response (Table 1).

The results obtained indicated that it would be possible to develop an experimental vaccine expressing FMDV empty capsids in transgenic alfalfa. However, the antigen concentration would need to be increased. The cassava vein mosaic virus (CsVMV) promoter presents a higher transcriptional activity in some alfalfa tissues than the CaMV35S promoter (Deborah Samac [leader of the Alfalfa Transformation Group at Minnesota USDA University], pers. comm., 2003). Transient expression assays performed by our group in alfalfa showed that the CsVMV promoter directed the expression of higher levels of the fusion protein p135–160-βGUS. Thus, this constitutive promoter appears to be an interesting tool to optimize transgene expression in alfalfa. We have recently produced transgenic alfalfa plants expressing P1 and 3C using

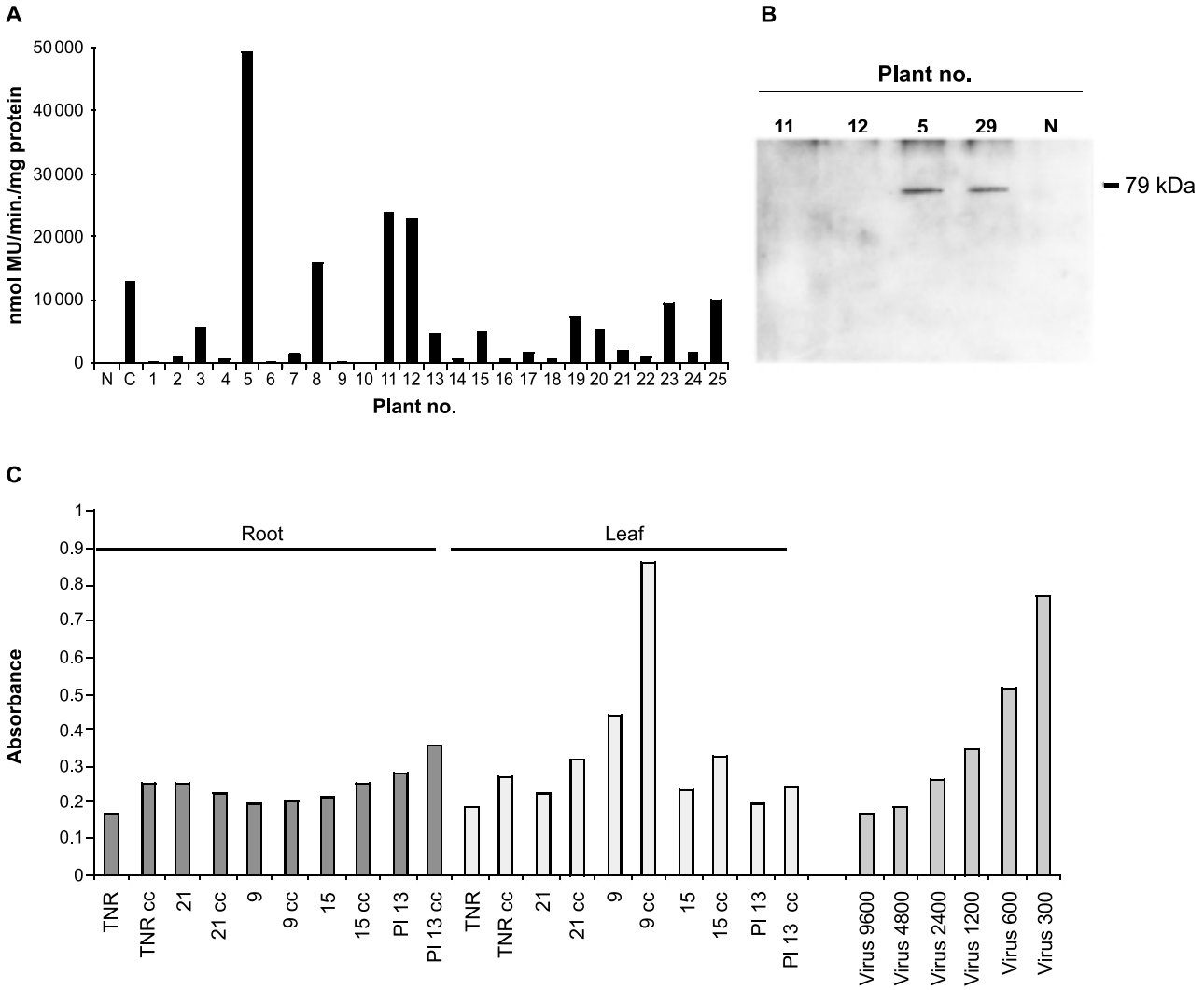


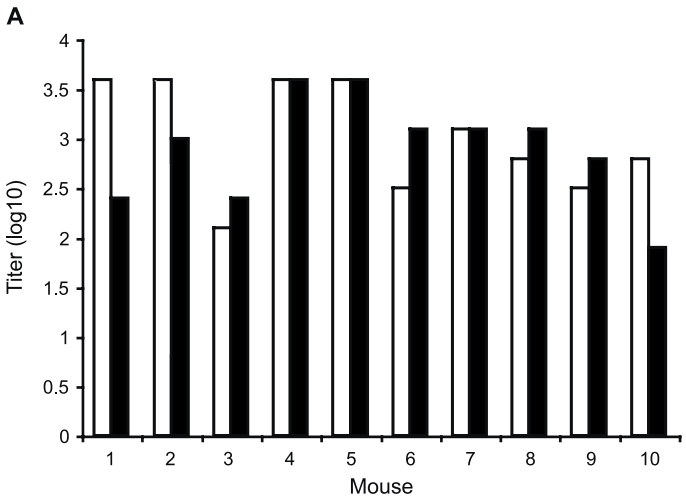
Figure 1 Detection of recombinant foot and mouth disease virus (FMDV) antigens on alfalfa transgenic plants. (A) Leaf extracts from different individual plants were tested for their enzymatic activity as described in the text. Each bar in the histogram represents the activity of individual transgenic plants (identified by numbers under the bars). N, plants transformed with a non-related gene; C, plant transformed with pBI121. (B) Detection of p135–160-βGUS expression in selected transgenic plants by western blot analysis using a mouse antiserum specific for p135–160. N, extracts from a plant transformed with a non-related gene. (C) Detection of P1-3C by a double antibody sandwich ELISA. Crude extracts of roots and leaves were concentrated three times with Amicom Ultra-4 Centrifugal Filter Devices (Millipore, Billerica, MA, USA). The approximate concentration of the recombinant protein was estimated by comparison with a known control preparation using quantified purified FMDV particles. TNR, plant transformed with a non-related gene; Cs, plant transformed with pROK-P1-3C using the CsVMV promoter; 35S, plant transformed with pROK-P1-3C using the CaMV35S promoter; cc, crude extract concentrated.

the CsVMV promoter. Preliminary results showed that it is possible to detect the foreign protein in 40% of transgenic plants, presenting a maximum level of expression of 0.6 μg/g fresh leaf.

Bovine rotavirus

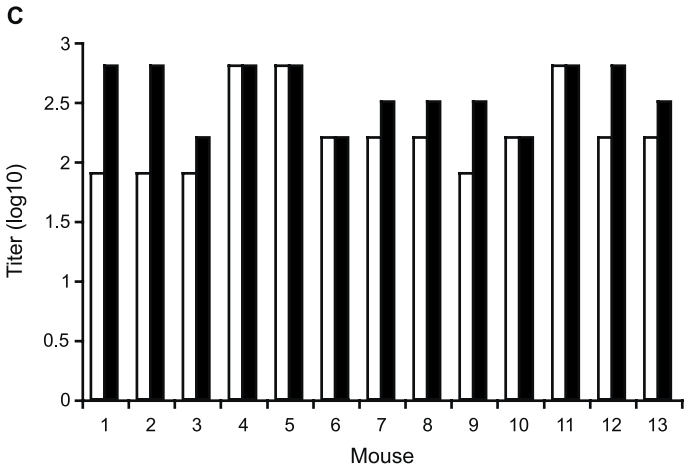
Because *eBRV4* is one of the few peptides capable of eliciting a protective immune response, we fused its coding sequence to the *gus* A gene in order to produce a fusion protein (*eBRV4*-βGUS) and expressed this product in transgenic alfalfa plants, in a similar way to what we used for the expression of FMDV p135–160. A 56 base pair (bp) DNA fragment (*eBRV4*) encoding the 232–242 portion of VP4 from BRV strain C486[41] was fused to the *gus* A gene. In addition, the native VP4 DNA sequences were adapted to the more frequent *A. thaliana* plant codon usage.[38] Again, petioles and embryos of alfalfa were submitted to *A. tumefaciens*-mediated transformation *in vitro* and transformed plants were initially selected for their ability to grow in agar media containing kanamycin.

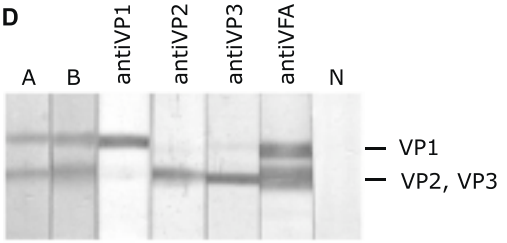
The βGUS activity was assessed in 62 transgenic individuals harbouring the *eBRV4*-βGUS gene. As expected, recombinant plants presented variable levels of βGUS activity, and approximately 4% of the individuals tested presented a significantly high level of expression of the βGUS protein



B

Plant transformed with	SNI
pBI121-p135–160 βGUS	2.1
	2.1
	1.8
	1.8
	2.1
	1.8
Non-related gene	<1.1
	<1.1





E

Plant transformed with	SNI
pROK-P1-3C (plant A)	2.2
	2.3
	2.1
	2.1
Non-related gene	<1.1
	<1.1

Figure 2 Immune response against foot and mouth disease virus (FMDV) induced by vaccination with transgenic alfalfa. (A) Detection of antip135–160 (□) and anti-FMDV particle (■) antibodies by ELISA in mice intraperitoneally immunized with leaf extracts from pBI121p135–160-βGUS-transformed plants. Numbers at the bottom of the bars correspond to different mice immunized with each of the different plant lines. Serum titres are expressed as the log of the reciprocal of the highest serum dilution that gives OD readings above the mean OD + three SD of sera from five animals immunized with plants transformed with a non-related foreign gene. (B) Neutralizing antibody response induced in vaccinated mice. Neutralizing index (NI) was determined as described in Dus Santos *et al*. (2002).[11] SNI, seroneutralizing index. (C) Detection of antibodies to p135–160 (□) and anti-FMDV (■) particles in mice intraperitoneally immunized with leaf extracts from pROK-P1-3C-transformed plants (plant A) using ELISA. Serum titres are expressed as in (A). (D) Anti-FMDV antibodies detected by western blot. A,B, different pools of sera from mice immunized with pROK-P1-3C-transformed plants (plant A). N, different pools of sera from mice immunized with plants transformed with a non-related recombinant gene. As a control, sera from mice specifically reacting with each of the FMDV structural proteins are included. (E) Neutralizing antibody response induced in vaccinated mice.

(Fig. 3A). Those plants presenting the highest βGUS activity (plant numbers 4, 10, 17 and 46) were analysed by western blot for association between βGUS activity and the expression of the *eBRV4* epitope fused to βGUS protein. Again, as in the case of the p135–160 epitope of FMDV, expression of the BRV epitope was effectively associated with the βGUS activity in each of the tested lines (Fig. 3B). The approximate concentration of the recombinant *eBRV4*-βGUS expressed in transgenic lines 17 and 46 was estimated to be between 0.4 and 0.9 mg/g TSP.

The immunogenicity of the plant-expressed *eBRV4* epitope was assessed in mice by parenteral or oral immunization with transgenic plant fresh leaves or extracts. Parenteral immunization of female mice was carried out as well as p135–160 epitope of FMDV using a pool of extracts from the *eBRV4*-βGUS-expressing plants 17 and 46. All mice vaccinated with the alfalfa-derived *eBRV4*-βGUS showed significantly higher antibody response than those immunized with the control antigen.

Animals fed with fresh leaves from plants expressing *eBRV4*-βGUS also developed a significant antirotavirus humoral response that was not present in control-vaccinated mice.

More interesting was the observation that the BRV antibody response elicited in a second group of dams immunized

Table 1 Protection of immunized mice against virus challenge

Plant transformed with	Route of immunization	Challenge virus	Protection (%)
pBI121-p135–160-βGUS	i.p.	FMDV (O1C)	100†
pBI121-non-related gene	i.p.	FMDV (O1C)	0†
pROK-P1-3C (plant A)	i.p.	FMDV (O1C)	100†
pROK-P1-3C (pool of other plants)	i.p.	FMDV (O1C)	90†
pROK-non-related gene	i.p.	FMDV (O1C)	0†
eBRV4-βGUS	i.p.	BRV (C486)	71‡
eBRV4-βGUS	Oral	BRV (C486)	76‡
Non-related gene	i.p.	BRV (C486)	21‡
Non-related gene	Oral	BRV (C486)	21‡

†Protection is expressed as no. protected mice/no. challenged mice after i.p. challenge 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.

‡Protection is expressed as no. protected mice/no. challenged mice after oral challenge with 10^4 focus forming units/mL. Protection is scored as lack of diarrhoea at 24 h post challenge.

BRV, bovine rotavirus; FMDV, foot and mouth disease virus.

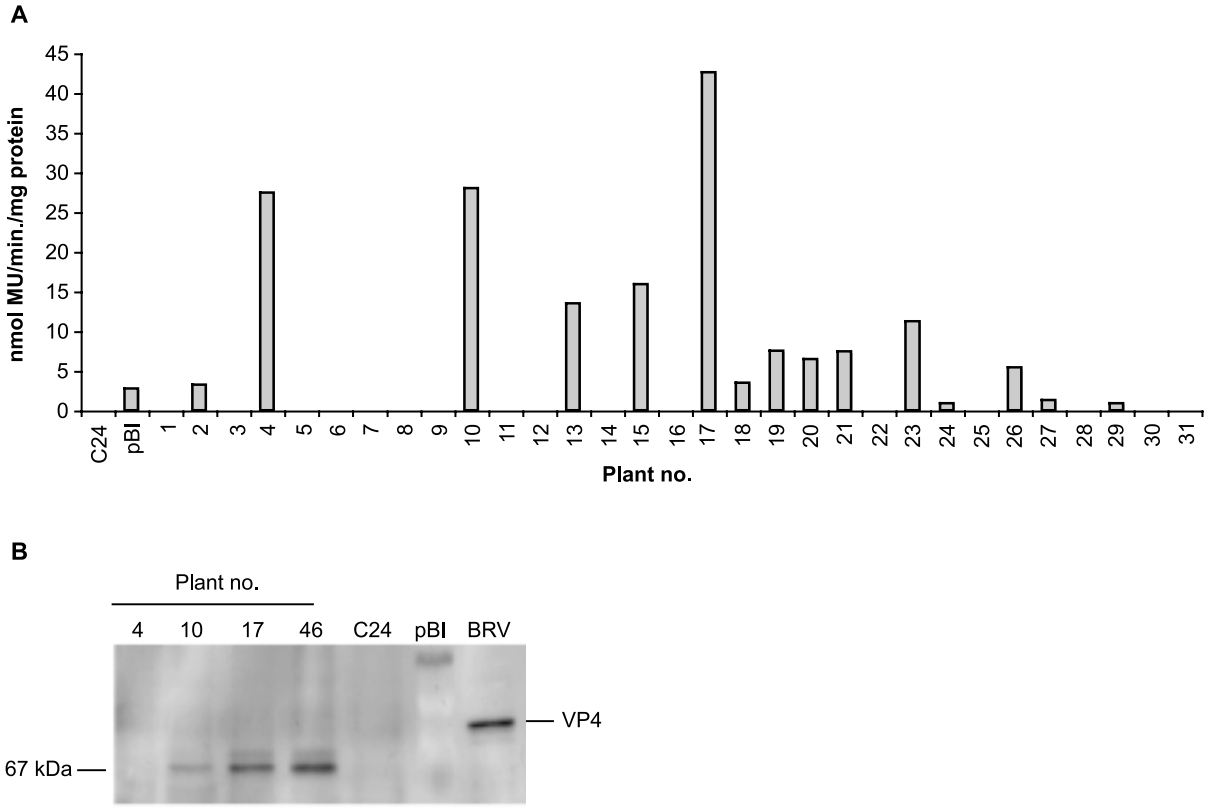


Figure 3 Detection of recombinant bovine rotavirus (BRV) epitope on alfalfa transgenic plants. (A) Detection of βGUS activity in the *eBRV4*-βGUS transgenic plants. C24, plants transformed with a non-related gene; pBI, plant transformed with the empty pBI121 vector. (B) Detection of *eBRV4*-βGUS expression in selected transgenic plants by western blot analysis using bovine polyclonal antibody against G6P1 rotavirus. BRV, purified rotavirus virus particles.

by either parenteral or oral routes was also passively transferred to their offspring (Fig. 4A,B). Pups born to parenterally or orally immunized dams showed levels of anti-BRV specific antibodies that were consistently positive, and they were protected against rotavirus challenge.

Challenge experiments demonstrated that regardless of the route of immunization of dams, all groups showed a significant degree of protection (Table 1).

Bovine viral diarrhoea virus

We produced plants containing the E2 gene under the CaMV35S promoter and TEV and SEKDEL regulatory signals. The recombinant binary vector, pBI121-TEV-Stag-E2-His-SEKDEL was developed in the context of the commercial vector pBI121 (Clontech), and was introduced into alfalfa as previously described for FMDV and BRV antigens. After seven

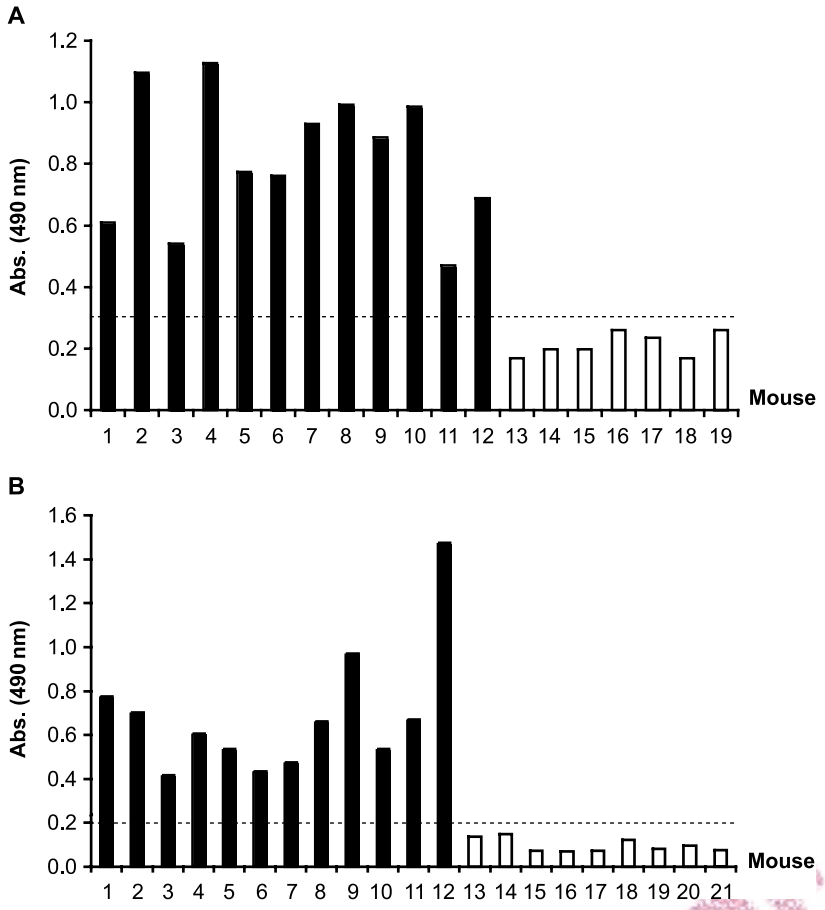


Figure 4 Immune response against bovine rotavirus (BRV) induced by vaccination with transgenic alfalfa. Detection of BRV antibodies in mice immunized with eBRV4-βGUS transgenic plants by ELISA carried out using p232–254 as antigen. (A) Mice intraperitoneally immunized with leaf extracts from pBI121*eBRV4*-βGUS-transformed alfalfa plants. (B) Mice fed with fresh leaves. ■, Readings from animals immunized with *eBRV4*-βGUS transgenic plants; □, readings from animals immunized with plants expressing a non-related antigen. Numbers at the bottom of the bars identify individual mice. Values are expressed as rough OD readings at 490 nm of a 1:20 serum dilution in PBS. Horizontal dotted lines indicate the mean OD + three SD of sera from animals immunized with plants transformed with a non-related antigen. Bars 1, 4, 7 and 10 represent sera from vaccinated dams while bars 2–3, 5–6, 8–9 and 11–12 represent sera of their respective offspring.

individual transformation events, 80 different transgenic plants with the ability to grow in the presence of kanamycin were produced.

The presence of the recombinant protein in the plants harbouring the foreign gene was investigated by ELISA and western blot. Using these methodologies, we could select two alfalfa plants with a high level of E2 expression (H6 and H26) (Fig. 5A,B).

The approximate concentration of the recombinant pBI121-TEV-Stag-E2-His-SEKDEL expressed in H6 and H26 transgenic plants was estimated to range between 0.05 and 0.5 mg/g TSP.

To characterize the selected plants, we determined the number of transgene insertions in the selected plants by Southern blot. The results obtained showed that plant H6 carrying three copies presented a higher protein level than plant H26 carrying five copies. Coincidentally with these results, mRNA levels observed in plant H26 were lower than in plant H6.

Discussion

Since the concept of using transgenic plants for vaccine production was first presented by Mason *et al.* (1992),[42] several authors have described the expression of vaccine antigens using this methodology.[1–5,7,9–12,43,44]

The demonstration that some of these antigens were immunogenic when orally administered[1–5,7,12] encouraged the study of other proteins and peptides expressed in plants in order to develop edible vaccines, particularly for those pathogens that may invade the organism through the oral route.

Although this expression system constitutes an interesting alternative to conventional methodologies, the main disadvantage is the low concentration of the expressed antigen. We have found the same inconvenience when we expressed viral antigens in alfalfa. Clear evidence of that was the difficulty in detecting the foreign protein in plant tissues as well as the necessity of multiple immunization doses to evoke an immune response.

This review shows the results obtained for the expression of immunogenic proteins from three viruses of veterinary importance: FMDV, BRV and BVDV. In addition, the immune response induced in experimental hosts was studied, as well as different strategies to optimize the levels of antigen expression in alfalfa transgenic plants.

Among the strategies used for increasing protein expression in alfalfa, we studied the generation of fusion proteins between immunorelevant viral epitopes and βGUS (p136–160-βGUS and *eBRV4*-βGUS), the use of alternative promoters (CsVMV promoter) and the optimization of genetic construction through the addition of regulatory sequences (TEV-L and SEKDEL).

Fusion of the p135–160 and *eBRV4* peptides to the reporter protein allowed us to simply detect the highest expressing plant lines by measuring their βGUS enzymatic activity, in accordance with previous reports that showed a positive association between βGUS activity and accumulation of the vaccine peptides fused.[12]

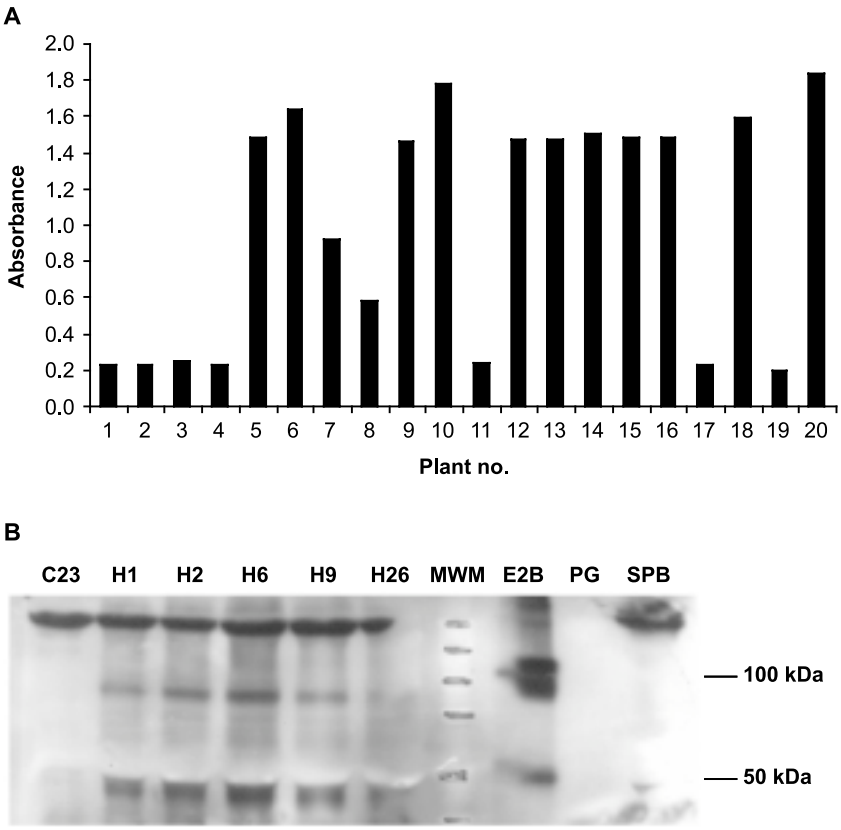


Figure 5 Detection of recombinant bovine viral diarrhoea virus (BVDV) E2 in alfalfa transgenic plants. (A) Detection of recombinant E2 in plants transformed with pBI121-TEV-Stag-E2-His-SEKDEL by a capture ELISA using a monoclonal antibody anti-E2. (B) Lines 1, 2, 6, 9, 26 (expressing E2) and C23 (expressing a non-related gene) were immunoprecipitated using a bovine polyclonal serum against E2. After immunoprecipitation, samples were assayed by western blot. E2B, E2 produced in insect cells as a positive control; PG, protein G; SBP, bovine polyclonal serum; MWM, molecular weight marker.

The level of the p135–160 fusion protein expressed in alfalfa was approximately 10-fold higher than the level observed in transgenic plants previously developed by us, expressing other recombinant proteins cloned in similar binary plasmids.[5,9,10] In concordance with this, the antibody titres reached by the immunized mice were markedly higher that those previously obtained in animals receiving complete VP1 expressed in alfalfa plants, which where not selected by their ability to produce high levels of the foreign protein.[5] Importantly, the FMDV epitopes included in p135–160-βGUS, expressed in transgenic plants as the p135–160-βGUS fusion protein, presented the same effectiveness in terms of inducing a protective antibody response as when administered as synthetic peptide.[8]

In the case of production of transgenic alfalfa expressing the *eBRV4* epitope from the structural protein VP4 of rotavirus fused to the βGUS protein, the results showed that plant-derived *eBRV4*-βGUS was able to induce a rotavirus specific antibody response in mice when administered either parenterally or orally. Notable was the observation that immunization of female mice induced not only an anti-BRV humoral response, but also a specific secretory antibody response in immunized dams, which they were able to transfer to their offspring through colostrum. Consequently, suckling mice presented specific rotavirus antibodies in serum at levels comparable to their mothers.

Importantly, the expression levels of epitope-βGUS appear very stable during the lifetime of a determinate plant as well as during the asexual reproduction cycles. This is a critical point to maintain a continuous and constant source of antigen.

As stated previously, most antigens expressed in transgenic plants are unique proteins or simple structures. HbsAg,[45] Norwalk virus capsid protein[46] and human papilloma virus capsid protein[47] are the only antigenic structures with some degree of complexity that have been stably expressed in plants. The practical use of transgenic plants as a source of antigen would be supported by the possibility of producing complex antigenic structures.

In line with this, we expressed FMDV polyprotein and protease 3C in transgenic alfalfa plants. Production of FMDV empty capsids requires proteolytic processing of P1 by protease 3C to generate the four viral structural proteins (VP1, VP2, VP3 and VP4) that self-assemble into empty capsids.

Inactivated vaccines against FMDV have proved to be effective tools for prevention of the disease. Although efficacious, their production is both costly and risky because manipulation of massive amounts of virulent virus could result in virus dissemination.[8] In addition, the immune response to the vaccine interferes with the ability to detect vaccinated animals that have subsequently become infected and could carry and shed the virus, creating an obstacle to reinstating disease-free status to countries that vaccinate to control outbreaks. Many diagnostic tests are based on the detection of antibodies to viral non-structural proteins that are present in low concentration in traditional FMDV vaccines and are poorly immunogenic in vaccine preparations. Expression of the empty capsid of the virus constitutes a feasible strategy to circumvent these disadvantages.[48]

Polyprotein P1 expressed in alfalfa evoked a strong neutralizing antibody response in an experimental model that was

able to protect immunized mice from viral challenge. Thus, protective epitopes were conserved in the product expressed in transgenic alfalfa plants. Preliminary results obtained by electronic microscopy showed spherical structures of 30 nm size in the transgenic plant transfected with P1-3C that were absent in a plant carrying a non-related gene. The identity of this structure is being confirmed by immunomicroscopy assays.

Expression of P1 and 3C in alfalfa suggested that FMDV empty capsids could be produced using nuclear transformation of alfalfa using *Agrobacterium tumefaciens*. However, the amount of antigen expressed was low and has no applicability for the development of an experimental vaccine. In order to circumvent this limitation, we are working on the optimization of expression levels in transgenic alfalfa. The utilization of the CsVMV promoter appears to be a feasible strategy to increase expression levels that undoubtedly will lead us to the development of an experimental FMDV vaccine based on the production of empty viral capsids.

In this review, we also show the results obtained for the expression of BVDV glycoprotein E2. Addition of the regulatory sequences (TEV-L and SEKDEL) allows us to detect the glycoprotein from leaf extracts in comparison with previous results expressing E2 without any regulatory signal. It is important to note that the protein expressed in plant tissues presented the same molecular weight as when it was produced in mammalian cells and insect cells, and a significantly higher molecular weight than the protein expressed in *Escherichia coli*. These results suggest that E2 expressed in alfalfa had post-translational modifications. This glycoprotein is now being used for immunization assays in experimental animals.

We believe that the results presented here support the concept of using transgenic plants as a novel and safe system for vaccine production, which could became an attractive alternative in animal health care. The fact that feeding animals with transgenic plants expressing a recombinant antigen caused a systemic immune response demonstrates the viability of using recombinant plants as both an expression and delivery system for oral vaccines, even against those antigens where a systemic immune response is required. The establishment of plant-based immunization methods for microbial antigens may be an inexpensive alternative to conventional fermentation systems for vaccine production, as plants can be grown inexpensively in large quantities and provide a practical and feasible delivery system.

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