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Expression of VP1 protein of serotype A and O of foot-and-mouth disease virus in transgenic sunnhemp plants and its immunogenicity for guinea pigs

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Summary. – Recently, transgenic plants expressing immunogenic proteins of foot-and-mouth disease virus (FMDV) have been used as oral or parenteral vaccines against foot-and-mouth disease (FMD). They exhibit advantages like cost effectiveness, absence of processing, thermostability, and easy oral application. FMDV VP1 protein of single serotype has been mostly used as immunogen. Here we report the development of a bivalent vaccine with tandem-linked VP1 proteins of two serotypes, A and O, present in transgenic forage crop *Crotalaria juncea*. The expression of the bivalent protein in the transgenic plants was confirmed by Western blot analysis. Guinea pig reacted to orally or parenterally applied vaccine by humoral as well as cell-mediated immune responses including serum antibodies and stimulated lymphocytes, respectively. The vaccine protected the animals against a challenge with the virus of serotype A as well as O. This is the first report on the development of a bivalent FMD vaccine using a forage crop.

Keywords: foot-and-mouth disease; sunnhemp; Agrobacterium tumefaciens; FMDV-VP1 gene; serotype O and A; in planta transformation; transgenic plants; bivalent vaccine

Introduction

FMD is one of the most devastating diseases of farm animals. Vaccination is the only pragmatic approach to control the disease in the endemic countries. Currently, vaccination against FMD is carried out using chemically inactivated FMD (Nagarajan, 2008). Though the conventional vaccine is effective in protecting animals from FMDV infection, its production involves high capital investment because of the need for sophisticated infrastructure requirement. Besides, it is risky to handle active virus in large scale for vaccine preparation as there are chances of virus leakage from production units (Brown, 1992). Further, the tissue culture-based whole virus vaccines are thermo labile needing cold chain maintenance during storage and transport. Also these vaccines cannot be used at the phase of outbreak as this will have a negative effect on immune response. Thus, it is necessary to develop newer vaccines that are safe, cost effective, can withstand normal physiological temperatures, and can be used during the outbreak. For vaccine use, it is necessary to produce protective antigens in heterologous host systems.

FMDV (the genus *Aphtovirus*, the family *Picornaviridae*) has positive sense RNA genome of 8.5 kb (Baranowski *et al*., 2003) that encodes both non-structural and structural proteins needed for virus replication and assembly respectively (Vakharia *et al*., 1987). The capsid is composed of 60 copies from each of the 4 structural proteins VP1, VP2, VP3,

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Abbreviations: FMD = foot-and-mouth disease; FMDV = FMD virus; VP1 A-O gene = linked gene of virus protein 1 FMDV of serotype A and O; VP 1 A-O protein = fusion protein consisting of tandem-linked VP1 proteins of two serotypes, A and O; SI = stimulation index; VNT = virus neutralization; p.v. = post vaccination; NC membrane = nitrocellulose membrane; i.m. = intra muscular

and VP4. Five antigenic sites have been identified on the capsid. Four of these antigenic sites involve one or more of the capsid proteins. Two immunogenic sites are located on serologically dominant VP1, where one of them is a major site present within the G-H loop (aa 140–160) and the other, a minor site located at aa 205–210 at C terminal end (Meloen and Barteling, 1986; Francis *et al.*, 1987). The peptides corresponding to aa 140–160 and 205–210 of VP1 indeed stimulate neutralizing antibodies in guinea pigs and cattle (Francis *et al.*, 1987). Hence, VP1 expressed in heterologous host systems has been exploited for the development of subunit vaccines (Suryanarayana *et al.*, 1992).

Deployment of plants as host systems for production of viral and bacterial antigens has been reported in the past (Dus Santos *et al.*, 2005). This strategy has several advantages such as, non-requirement of sophisticated fermentation and cumbersome downstream processing. The technology is user-friendly and besides that, it provides an opportunity for mass immunization as the edible parts of the plant can be directly fed (Khandelwal *et al.*, 2003). Moreover, oral administration of the vaccine stimulates immune responses at the mucosal surface and the integrated nature of the mucosal immune system allows other mucosal sites also to be primed (Ruedl and Wolf, 1995).

Recently, several plant species have been successfully used for expressing FMDV immunogens and have been studied as vaccines (Carrillo *et al.*, 1998; Dus Santos *et al.*, 2005; Pan *et al.*, 2008). FMDV exists as 7 antigenically distinct serotypes (A, O, C, Asia1, SAT1, 2, and 3) and several antigenically distinct strains with no cross protection among serotypes (Ogra *et al.*, 2001). Thus, vaccines to be used in a particular geographical area should contain the antigenic moieties corresponding to the circulating strains. In endemic countries, polyvalent vaccine containing a mixture of circulating dominant serotypes selected by antigenic coverage, is used as prophylactic vaccine. Thus, any alternate vaccines for these settings should contain polyvalent immunogens. So far the studies have been directed towards the production of major immunogen, VP1 alone (Carrillo *et al.*, 1998) or VP1 in combination with other capsid proteins (Pan *et al.*, 2008) of a single serotype in plants. Till now, no attempts have been made to study the immune response to a complex antigen consisting of several immunoreactive proteins. Therefore, in this work, we attempted to prepare transgenic forage crop, sunnhemp plants expressing FMDV VP1 proteins of two serotypes, O and A linked in tandem, and tested them as bivalent FMD vaccine in guinea pig model.

Materials and Methods

Virus. FMDV serotype O (Ind R2/75) and A (Ind 17/77) vaccine strains at 5th passage level maintained at FMD vaccine production unit (I.V.R.I., Bangalore) were used. The viruses were passaged once in BHK-21 (Clone 13) monolayer cells.

*Plasmid construct***.** Total RNAs from O and A virus-infected cell culture supernatants were extracted with the Trizol reagent (Invitrogen, USA). The cDNA was synthesized from the total RNA using oligo d(T) primer as in standard protocol (Sambrook, 2001) and used as template for amplification of the desired FMDV VP1 gene with serotype-specific primers. For amplifying VP1 O we used, O Kp.Sp.Ml (L): 5′-GCG GGT ACC GCA TGC GGA CGC GTG TAT GAC CAC CTC CCC GGG TGA G-3′ and O *Bam*H1(R): 5′-GCG GAT CCG CCG GGG GTT CAG GAG CTG CTT-3′ primers, and for VP1 A: A *Bam*HI(L): 5′-GGC GGA TCC AGA CCA CCG CTA CCG GGG-3’ and A *Eco*RI(R): 5′-GCG AAT TCG CCG GGG TTC AAA AAC TTG CTT CTC AGG-3′ primers.

VP1 gene of serotype O followed by serotype A (1,3 kb) in 5′–3′ direction were cloned in to pcDNA vector. The insert was then subcloned in to pBSK^{+} vector carrying CaMV35S promoter. The entire cassette (CaMV35S-VP1O-A) was transferred to pCAMBIA 2301 (Hajdukiewicz *et al.*, 1994). This has resulted in the replacement of the GUS gene in the original pCAMBIA 2301vector with the CaMV35S-VP1O-A. The recombinant vector pCAMBIA VP1 was transferred into the *Agrobacterium* host LBA4404 by electroporation (Gene Pulser, Bio-Rad).

Transgenic sunnhemp plants. Agrobacterium-mediated *in planta* transformation method for development of transgenic sunnhemp plants was described elsewhere (Rao *et al.*, 2012). The germinating seeds of sunnhemp (*Crotalaria juncea* L. C.V. Bellary) were infected with *vir* gene-induced (Cheng *et al.*, 1996) *Agrobacterium* host LBA4404 harboring the recombinant plasmid, pCAMBIA VP1 O-A. The seeds of these putative transformants were used for raising the next (T_1) generation.

PCR screening of putative transformants of T1 generation. The genomic DNA from the fresh leaf tissues of the plants was extracted by the C-TAB method (Doyle and Doyle, 1987). The presence of VP1 O-A construct and the marker gene *Npt*II (700 bp), was detected by PCR. The primers for amplifying FMDV specific insert were described earlier. For amplifying *Npt*II, primers *Npt*II: 5′-GAG GCT ATT CGG CTA TGA CTG-3′ and *Npt*II: 5′-ATC GGG AGG GGC GAT ACC GTA-3′ were used.

*RT-PCR***.** Presence of the RNA transcripts in the PCR positive plants was analyzed by RT-PCR as in the standard methods. The total RNA from the positive plants was extracted using the HiPurA^{TM} plant RNA miniprep purification spin kit (Hi media, India). Total RNA (1 μg/10 μl) was used to synthesize the cDNA using Superscript III as in manufacturer´s protocol (Invitrogen, USA) and used as a template for amplifying specific genes (650 bp VP1 and 750 bp NptII).

Extracts of transgenic plants. The total proteins from the putative transgenic plants were extracted as per the standard TCA-acetone method (Méchin *et al.*, 2007). Quantity of the total protein was estimated by Bradford method (Bradford, 1976).

Western blot analysis. The total proteins (200 μg) from the putative transformants were subjected to discontinuous 10% SDS-PAGE

as described by Laemmli *et al.* (1970). The separated proteins were electro-transferred on to a nitrocellulose membrane (Millipore, India). The NC membrane was incubated in 5% milk for 1 hr at 37°C, followed by incubation with rabbit anti-FMDV antibody raised against *Escherichia coli* expressed, affinity purified bivalent protein, for 1 hr at 37°C. After washing, the blot was incubated with goat anti-rabbit IgG-HRP conjugate (Sigma, USA) and developed with OPD substrate (Sigma) as described by Suryanarayana *et al.* (1992).

ELISA. Quantity of the expressed bivalent protein was estimated by ELISA, using specific antiserum raised against purified bivalent protein and purified *E. coli* expressed protein. The protein extracts from the positive plants were diluted 5-fold and 100 µl of the diluted protein was applied to 96-well plates and left at 4°C overnight. After washing, the unbound sites were blocked with 3% milk. The plates were washed and anti-rabbit hyper immune serum (1:2,000) was added. Detection was done by goat anti-rabbit IgG-HRP conjugate (1:1000) followed by chromogen OPD. The absorbance was measured at A_{492} in ELISA reader.

For ELISA of serum antibodies, 1 µg of the *E .coli* expressed purified bivalent VP1 protein was coated on to 96-well plates at 4°C overnight. Pooled test sera of each vaccinated group in two fold serial dilutions (1:20 to 1:160) were used.

Immunization of guinea pigs. Thirty male guinea pigs with body weights of 400–500 g were randomly divided into five groups of six animals. Animals in group A were immunized with 50 µg of *E. coli*-expressed protein with equal volume of Freund's incomplete adjuvant in to gluteal muscle; group B animals were immunized with leaf extracted proteins corresponding to 1 g of the leaf material from transgenic plants and mixed with Freund's incomplete adjuvant; group C animals were fed with 1 g of leaves of the transgenic plants; group D animals were immunized with 0.2 ml of inactivated FMD vaccine; group E animals were immunized with extract of 1 g of the control plant leaves as negative control. Booster dose was given on 21st day post vaccination (p.v.). Animals were bled at day 0, 14, 21, and 28 p.v.

Lymphocyte stimulation assay. The whole blood lymphocytes were collected from the vaccinated animals on day 14, 21, and 28 p.v. (Boyum, 1968). The cell density was adjusted to $2x10^6$/ml, dispensed into 96-well plates and the lymphocyte stimulation assay was performed as in the standard protocol. Absorbance was measured at 630 nm in ELISA reader and stimulation index (S.I.) was calculated.

Virus neutralization test (VNT). Complement inactivated test sera were diluted serially two fold from 1:8 to 1:128 in Eagle's MEM and 50 µl of each dilution was delivered into 96-well plates in triplicates. Fifty µl of 100 $TCID_{50}$ of FMDV O was added to each well and incubated at 37°C in a humidified chamber with 5% CO_2 for 1 hr. Fifty µl of BHK-21 (Clone13) cell suspension ($0.5x10^6$ cells /ml) was added to each well and incubated for 48 hrs in a CO_2 incubator at 37°C. The log serum dilution at which 50% of the monolayer showed cytopathic effect was taken as a titer.

Protection of guinea pigs against FMD. On the 36th day p.v., the guinea pig groups were separated, each animal in the group was challenged by injecting 100 µl of 100 $GPID_{50}$ guinea pig adopted FMDV O or A in the left rear footpad and examined for the appearance of lesions for 7 days. The absence of secondary lesions (on the uninoculated foot pads) was considered as an indicator of protection (Yu and Cui, 1997).

Results and Discussion

Many viruses select mucosal surface for infection and most of these diseases are highly contagious. FMD of cloven-footed animals is one of such most feared animal diseases over the world and the control of this disease is of priority for both, endemic and disease-free countries, as the presence of the virus in any part of the globe is considered unsafe to the rest of the world. In endemic countries like India, regular vaccination is the pragmatic approach. Vaccines against FMD are prepared with the viruses grown in BHK-21 cells after inactivating with chemical inactivants. Since FMDV exists in multiple serotypes and strains, the vaccine to be used in any geographical region should contain all the serotypes that are in circulation in that area. The conventional cell culture based vaccines have several limitations which include, risk of virus handling in bulk and thermal instability of the tissue culture based vaccines. Further, these vaccines cannot be used at the phase of outbreak. The alternative approach could be to produce potent immunogen in heterologous host systems for using both, in parenteral and mucosal administration.

In this respect plants can be considered as ideal host systems, as they are environmentally friendly and their edible parts can be used as a vaccine with no further processing. Among plant species, forage legumes are ideal for developing edible vaccines against animal diseases like FMD (Dus Santos *et al.*, 2002, 2005) and rinderpest (Satyavathi *et al.*, 2003; Prasad *et al.*, 2004), as the foliage of these crops is used as protein rich fodder. With this objective in mind, we selected sunnhemp (*Crotalaria juncea*) for expressing the bivalent FMDV genes. Among the FMDV serotypes that are in circulation in India, O occupies first place with the highest number of outbreaks (>60%) followed by A serotype. Hence, we selected the immunogenic protein genes of these two serotypes for our studies.

Since, the cotyledonary nodes which are highly meristematic were targeted for transformation, the resulting plants from this event will be chimeric. Therefore 60 putative transformants from three independent transformation events (T_0) were grown and the seeds from these plants were sown to get 180 T_1 plants. The presence of FMDV VP1 gene and its expression was studied in these plants.

Analysis of T_1 transgenic plants

The T_1 plants were screened for the integration of DNA of the recombinant binary vector, pCAMBIA carrying VP1

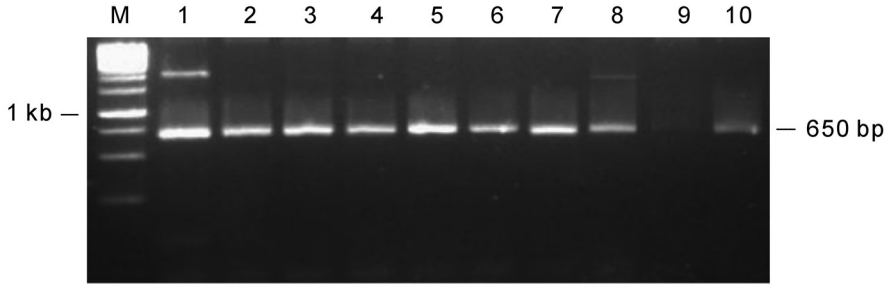

Fig. 1

Screening of transgenic plants by PCR for FMDV VP1 O-A gene

Agarose gel electrophoresis of PCR products. Positive plants (lanes 1–8), negative control plant (lane 9), p CAMBIA-2-VP1 positive control (lane 10), DNA size marker (lane M).

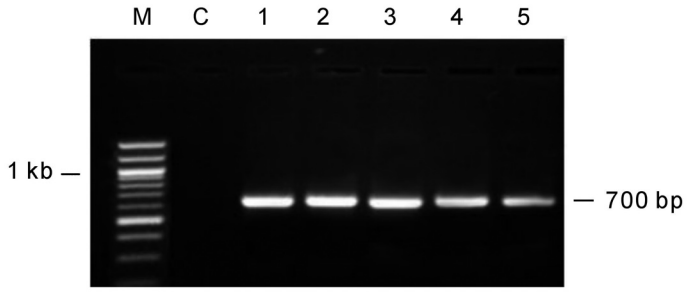

Fig. 2

Screening of transgenic plants by PCR for marker *Npt*II gene

Agarose gel electrophoresis of PCR products. Positive plants (lanes 1–5), negative control plant (lane C), DNA size marker (lane M).

O-A into genomic DNA of individual plants. PCR was carried out using VP1 and *Npt*II specific primers and the products were analyzed by agarose gel electrophoresis (Figs. 1, 2). As shown in the Fig.1 (lanes 1–8) amplification of 650 bp fragment corresponding to VP1 of either O or A can be seen. Since the primers correspond to conserved sequences it is expected that both VP1 genes can be amplified with a single set of primers. There was no amplification for the bivalent gene in the DNA from control plant (lane 9). This indicates that the transformants carry VP1 gene of O, A or both serotypes. The size of the amplicon was in good agreement with the amplification in the positive control (pCAMBIA-2-VP1 DNA) (lane 10).

The plants, positive for the presence of VP1 O-A were also screened for the presence of marker gene *Npt*II (700bps). The Fig. 2 shows amplification of 700 bp fragment corresponding to *Npt*II (lanes 1–5) while the wild type plants (lane C) did not show any amplification. It was observed that some plants showed the presence of only one marker. Similar observation was made by Atkinson and Gardner (1991, 1992), Yao *et al.* (1995), and Rohini and Sankara Rao (2000). The 18 of 180 T_1 plants positive for the presence of the antigenic gene VP1 were selected for further screening. The PCR positive plants were further screened for the presence of the bivalent VP1 mRNA in the total RNA population by RT-PCR. Twelve of 18 positive plants screened by PCR showed the presence of FMDV specific transcript indicating that the inserted gene was expressed in these plants. Fig. 3 shows amplification of 650 bp fragment corresponding to VP1 gene in 6 genomic PCR positive transformants (lanes 1, 2, 4, 5, 6, and 8). The size of the fragment corresponded to the VP1 gene that was amplified from the cDNA of the FMDV O serotype (lane 10). There was no amplification observed in 2 genomic PCR positive plants (lanes 3, 7) and 1 uninfected control plant (lane 9) indicating that the amplification was specific. The absence of the transcript in 2 plants can be attributed to events such as gene silencing or DNA methylation. It has been suggested by other studies that gene silencing and DNA methylation are positively correlated to expression of the transgene (Desai, 2010; Veluthambi *et al.*, 2003). False positive results were ruled out by performing direct PCR of the RNA samples (not shown). There was no amplification when the extracted RNA was subjected for PCR indicating that there was no genomic DNA in the preparation.

The positive transformants confirmed by RT-PCR analysis were further subjected to Western blot analysis. The total

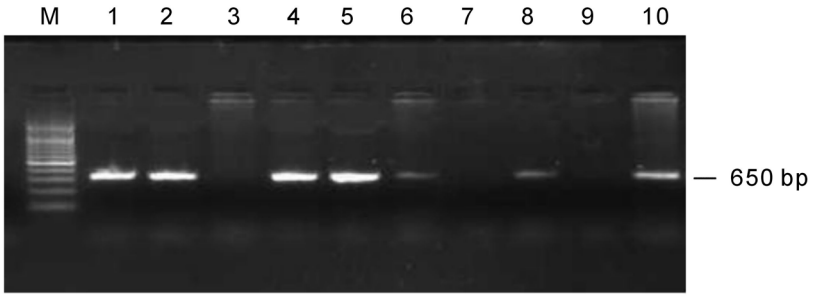

Fig. 3

Screening of transgenic plants for expression of FMDV VP1 O-A mRNA

Agarose gel electrophoresis of PCR products of RT-PCR. Positive plants (lanes 1, 2, 4, 5, 6, 8), negative plant (lanes 3, 7), negative control plant (lane 9), cDNA of FMDV O serotype (lane 10), DNA size marker (lane M).

protein extracted from the 12 positive transformants and 1 negative plant was subjected to 10% SDS-PAGE. The proteins resolved by SDS-PAGE were transferred onto NC membrane and immunodetected. A single intense band of 48 kDa was detected in 7 plants of 12 RT-PCR positive plants which confirmed the expression of the bivalent VP1. Fig. 4 shows expression of the bivalent VP1 in 4 positive transformants confirmed by RT-PCR analysis (lanes 1, 2, 3, 4). The size of the expressed protein is 48 kDa and is in good agreement with the native protein expressed in BHK-21 infected cells. Since the protein reacted with the hyper immune serum, it can be concluded that the protein carries the major immunogenic epitopes defined by G-H loop of VP1 and may be in native conformation which could be further confirmed by animal protection studies.

As the integration of the gene in *Agrobacterium* mediated transformation is a random event (Jones *et al.*, 1985), the expression levels may vary amongst the plants. The quantity of the bivalent FMDV antigen VP1 O-A expressed by each of the 7 transgenic plants was estimated by indirect ELISA using purified *E. coli* expressed protein as standard. The amount of bivalent VP1 in the total soluble protein was estimated from the standard curve and ranged from 1 μg/g to 12 μg/g of soluble protein.

Of the seven independent transgenic plants the maximum expression was observed in 24th and 37th transgenic line suggesting that these lines have a copy number which is optimum for high expression or the position of the gene integration such that it is favorable for high expression. However, a better way of confirming it would be by Southern blot. Expression of the heterologous proteins by constitutive promoters like CaMV35S is subjected to several regulatory elements and may reflect differential expression in different tissues of the same plant (Benfey and Chua, 1990). Hence, modifications like use of tissue-specific promoters, gene codon optimalisation (Gustafssion *et al.*, 2004), and co-expression of the transgene with protease inhibitors can be tried for enhancing and stabilizing expression. These two independent transgenic lines were selected and protein samples were pooled to immunize the guinea pigs. Though the transformation efficiency in our experiments was found to be 3%, use of host strains like EHA105 with higher copy number of vir G gene (Hood *et al.*, 1993; Meurer *et al.*, 1998) may help to increase the efficiency.

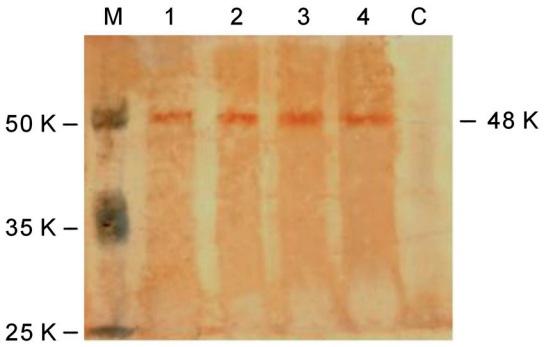

Fig. 4

Screening of transgenic plants for expression of FMDV VP1 O-A protein

Western blot analysis: Positive plants (lanes 1–4), negative control plant (lane C), protein size marker (lane M).

Immunization of guinea pigs with the vaccine

The pooled sera samples of experimental animals were tested for the presence of specific antibodies by indirect ELISA using *E. coli* expressed recombinant bivalent VP1 as antigen. Serum titers were expressed as the log 10 of the highest dilution that represents OD readings above the unvaccinated control animal group (Fig. 5). Antibody titers of group A, B, C, and D reached the highest point from the

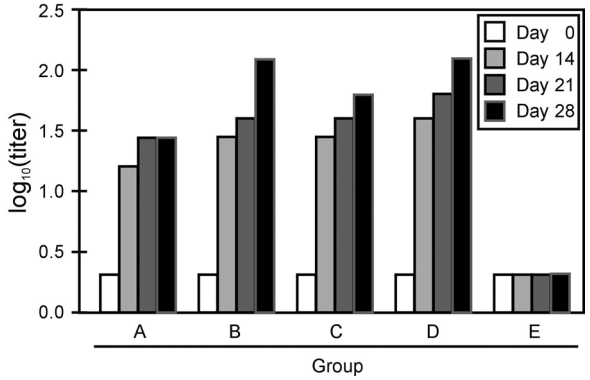

Fig. 5

Specific antibody titers in animals immunized with the vaccine

ELISA log titers at days 0–28 p.v. Groups A-E are explained in the Table 1.

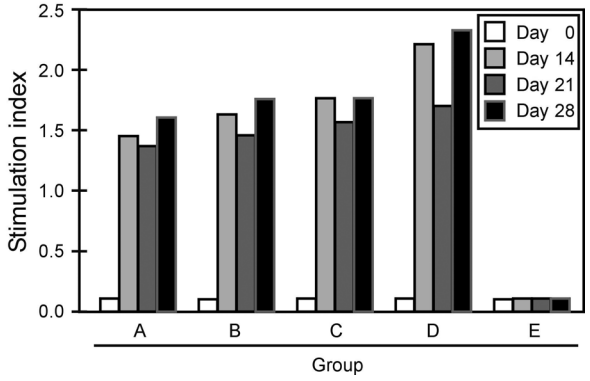

Fig. 6

Lympho-proliferation assay of lymphocytes isolated from immunized animals

The results are expressed as stimulation index. Stimulation index more than 1.5 is considered positive. Groups A-E are explained in the Table 1.

14th to 21st day. This is in good agreement with the findings reported by Abu Elzein *et al.* (1981). Because of the booster dose on the 21st day, titers of group B (parenteral vaccinated group) increased from 1.6 to 2.10. Interestingly, the oral-fed group C has also shown an increase in the titer (from 1.6 to 1.8) indicating FMDV specific humoral immune response. There might also be an increase in FMDV specific secretary IgA antibody response as this was expected with oral immunization in case of mucosal vaccines produced in leguminous plants. It has been reported from the studies that *Crotalaria* lectins act as adjuvants (Kristiansen *et al.*, 1983). This is also clearly supported by previous findings of plant constituents capable of stimulating antigen specific and non-specific immune responses (Bae *et al.*, 2003; Guo *et al.*, 2000; Puri *et al.*, 2000; Daswani *et al.*, 2002; Kim *et al.*, 2002a,b). However, the contribution made by mucosal antibodies can be assessed only by detecting isotypes as reported by Doel *et al.* (1996). Similar to the B and C group, the antibody titers of conventional vaccine group also increased to the highest point after booster. There was no significant increase in the titers of group A (*E. coli* expressed purified protein) after booster. This may be due to poor immunogenicity of protein produced in bacterial system because of the unfavorable conformation. Poor immunogenicity of the bacterially expressed VP1 has been reported by several workers (Suryanarayana *et al.*, 1992). Between the two plant experimental groups, it was observed that group B had slightly higher titers than the group C on all three days. Similar results were also reported by Fabienne *et al.* (2003). The reason may be due to the route of administration. Since the oral vaccines induce immune response in mucosal surface and the antigen is given as a crude total protein, it may be difficult to assess the quantity of antigen taken up by the immune system. Hence, dose response studies may be needed to know the amount of foliage to be fed for optimum response. However, as in quantitative ELISA, the amount of antigen fed to a guinea pig comes to 12 μg (from 1 g of foliage), which is much less when compared to the *E. coli* expressed protein used for immunization by several workers indicating the expressed protein showed good immunogenicity, that may be due to favorable conformation. Sometimes chewing and digestion may cause degradation of the antigen (Chen *et al.*, 2006). Use of parenteral priming of mucosal vaccines can be an alternative and help in development of better immune responses in this group.

Table 1. Guinea pig protection studies

Groups	Immunization scheme	Protection against (%)	
		O	A
A	*E. coli* expressed VP 1 O-A protein, i.m. application	66	33
B	Proteins from transgenic plants, i.m. application	66	66
C	Transgenic plants, oral application	66	66
D	Inactivated FMD vaccine, i.m. application	100	100
E	Proteins from normal plants, i.m. application	0	0

i.m. = intra muscular application.

Presence of virus neutralizing antibodies in vaccinated animals

The ability of the test serum to neutralize FMDV infection *in vitro* on BHK-21 cells was checked. Serum of the control group (unvaccinated animals) displayed no neutralizing ef-

fect against FMDV, whereas on the 28th day p.v. the serum of the group B and C significantly neutralized FMDV with the average titers being 1.8 and 1.5, respectively and resulted in no CPE in BHK-21 cells. The positive control group D, showed the highest titers of 2.10. Group A showed the least titer compared to the three other groups (data not shown). The fecal and mucosal antibodies, IgG and IgA were not used to check the neutralizing effect as it would be difficult to collect samples and separate isotypes. Although, it is reported that these isotypes are more ideal to assess VNT (Chen *et al*., 2006) as they are induced after oral delivery.

Stimulation of lymphocytes from vaccinated animals

The stimulation of lymphocytes of all the four groups showed a general trend of a peak of S.I. on the 14th day followed by decline on the 21st day and again a steady increase on the 28th day (Fig. 6). This is in agreement with the findings of Knudsen *et al*. (1979). There was no much significant difference in S.I. of group B and C. However the S.I. was 3 fold higher than the control group E, but less than the S.I. induced by the conventional vaccine group D. The group A showed the least S.I. when compared to all other groups. Since the major effector cells of the mucosal surfaces are T cells of both T_4 and T_8 phenotypes (Conley *et al*., 1987) which in turn produce IgA antibodies. From our results of MTT assay, we can hypothesis that there is development of mucosal immune response at least in group C which was orally fed.

Protection effect of the vaccine

All the animals were challenged on the 36th day p.v. with 100 $GPID_{50}$ of FMDV serotype O or serotype A. The animals were observed for development of secondary lesions consisting of vesicle formation on the right footpad. Two of the 3 animals in the *E .coli* expressed protein-vaccinated group namely group A could withstand challenge with O virus (66%) while 1 of 3 could survive challenge with A (33%). While both the plant experimental groups B and C (injected and orally fed groups) showed 66% (2/3) protection for both serotypes. Animals in the conventional vaccine group D showed 100% protection for both O and A serotypes. The conventional vaccine group D performed better when challenged, as it could induce better immune response due to the presence of conformational epitopes of the capsid proteins, whereas the other three groups A, B, and C carry only 2 sequential epitopes which may not be sufficient for protecting animals against FMDV by needle challenge. All animals in the control group developed clinical signs of FMD and developed secondary lesions on tongue and feet within 2–4 days after challenge. Since these studies are to show the scope of using plant expressed proteins as mucosal vaccines for use at the phase of disease outbreak, the challenge experiments are to be conducted with aerosol virus. Since the method of challenging guinea pigs with aerosol virus is not available, it may be ideal to use bovine calves or pigs for such studies.

Our results in the present study support the possibility of using forage legume crop, sunnhemp for production of the FMDV bivalent VP1 O-A antigen. The methodology can be employed for transformation of other forage crops such as mung bean (*Phaseolus aureaus),* poor man's bean *(Dolichos lablab),* egyptian clover (*Trifolium alexandrinum*), and peanut (*Arachis hypogaea)* which are widely used for fodder. We have demonstrated that the plant expressed FMDV bivalent VP1 O-A antigen is capable of inducing neutralizing antibody responses and protection from FMDV challenge in guinea pigs. Most importantly, although no definite data is shown, our results suggest the development of mucosal vaccine via plant expression systems. However, though the animals showed good responses, it is not comparable to immunization with conventional vaccine group. This suggests that it is necessary to optimize the route of vaccine administration, increase the number of booster dosages or use mucosal adjuvants etc. We are also focusing on extrapolating our findings by studies of immune responses for plant expressed FMDV bivalent VP1 O-A antigen in cattle which are the actual FMDV hosts.

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