Tissue culture independent transformation of the forage crop sunnhemp (*Crotalaria juncea* L.): an easy method towards generation of transgenics

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Abstract A transformation system which is free of *in vitro* plant regeneration following *Agrobacterium* infection is established for the forage legume, Sunnhemp (*Crotalaria juncea* L.) where in the entire embryo axis of the germinating seed was used as the target tissue for transformation. After standardization of transformation conditions, the cotyledonary node of the embryo axis was infected with *Agrobacterium* host LBA 4404 harboring the recombinant vector pCAMBIA 2301. The bivalent 1D gene of the two major foot and mouth disease virus (FMDV) serotypes ‘O’ and ‘A₂₂’ and the neomycin phosphotransferase (nptII) gene were used as the markers for optimization of the protocol. The embryo axes were pricked randomly on the cotyledonary node and co-cultivated with *Agrobacterium*. The germ-lings were then allowed to grow under standard growth

room conditions in to mature fertile plants. 60 T₀ plants were established from 3 separate experiments. Three hundred seeds from the 60 T₀ plants were sown to raise the T₁ generation of which 180 were analyzed for integration of bivalent FMDV gene 1D “O” and “A₂₂” and the nptII gene. Eighteen out of these 180 plants amplified both the marker genes. Two independent transgenic lines 24 and 37, showed elevated levels of expression of 12 µg and 8 µg (per gm of fresh leaf) of the bivalent ID antigen “O” and “A₂₂”. The results showed that the transformation efficiency was 3 %. To the best of our knowledge, this is the first successful attempt of *Agrobacterium tumefaciens* mediated transformation of Sunnhemp. The protocol can generate whole plant transformants with relative ease and should be compatible to all genotypes of Sunnhemp.

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Introduction

Till recently legumes have been branded as recalcitrant for transformation, as regeneration *in vitro* is highly genotype specific and requires a lot of skill. Besides the frequency of transformants generated was very low to make any significant impact. Thus the need of the hour is to develop techniques which used the totipotent cells of the legumes to generate transformants. The most popular and user friendly amongst the recent techniques is the *in planta* transformation by *Agrobacterium tumefaciens*. A number of legumes (Trieu et al. 2000; Olhoft et al. 2001 etc.) have been transformed by this procedure. These developments have further enhanced

the importance of forage legumes for exploitation in development of edible vaccines for immunization against animal diseases like Rinderpest disease (Prasad et al. 2004), foot and mouth disease (Dus Santos et al. 2005, 2002) as these crops are generally used as protein rich fodder.

Crotalaria juncea commonly known as Sunnhemp belonging to the *papilionaceae* family originated in India (Purseglove 1981). Species of this genus are wide spread throughout tropical, subtropical and to lesser extent temperate countries. It is used as forage for cattle owing to the large amounts of water soluble gums and proteins in their seeds (Pandey and Srivastava 1990). Although there are few reports of regeneration *in vitro* earlier, (Ohara et al. 2000) the viability of transgenics of this plant has not been exploited as it is a legume.

In the present study, we have looked at the possibility of using a forage variety of Sunnhemp (*Crotalaria juncea*) for expressing the bivalent FMDV genes. The FMDV viral capsid consists of 60 copies of 4 structural proteins termed as VP1 (1D), VP2 (1B), VP3 (1C) and VP4 (1A). The immune-dominant epitopes are located on the 1D with in the G-H loop (140-160aa) and C terminal end (205-210aa) (Meloan and Barteling 1986). Thus 1D gene has been exploited as the ideal candidate for development of edible vaccines (Carrillo et al. 1998) for foot and mouth disease (FMD). FMDV exists as 7 antigenically distinct serotypes (O, A, C, Asia 1 and SAT 1–3) with no cross reactivity among them (Ogra et al. 2001). In India 3 of them (O, A, Asia1) are in circulation and 2 of these ('O' and 'A') are responsible for large number of outbreaks. Hence vaccines against these are necessary to avoid major economic losses. With this objective in mind we have selected 1D gene of FMDV serotypes "O" and "A" in the present study.

Materials and methods

Recombinant vector and bacterial hosts pCAMBIA vector 2301 derived from the pPZP vectors (Hajdukiewick et al. 1994) were used in this experiment. The generally employed GUS reporter gene is not ideal for legume transformation as the legumes show an endogenous GUS like activity. For this reason, we have used another gene construct namely, the bivalent 1D gene of the two major FMDV serotypes 'O' and 'A22' as one of the markers. (The detailed account of immunization studies is being published elsewhere). The original pCAMBIA 2301 vector was modified by replacing the GUS gene with the bivalent 1D FMDV so that it is under the control of the CaMV35S promoter. The recombinant vector also has pVS1 replicon and the nptII gene driven by CaMV35S promoter as the plant selection marker (Fig. 1).

The disarmed *Agrobacterium* host LBA 4404 was transformed with the recombinant vector pCAMBIA 1D by

electroporation (Gene Pulser, Bio-Rad) (Wen-Jun and Forde 1989). The presence of the recombinant vector in *Agrobacterium* was confirmed by restriction digestion of the plasmid isolated from the transformant

The T-DNA of the pCAMBIA 2301 contains neomycin phosphotransferase gene under the constitutive promoter CAMv 35S and NOS (nopaline synthase) poly A terminator. Following this is a polylinker region downstream to which is the GUS gene again under the control of CaMV 35S promoter followed by NOS poly A termination site. The GUS gene is replaced by the CaMV 35S -1D at the Sal and BstEII sites in the recombinant vector pCAMBIA 1D (12.16 Kb)

Explant material Seeds of Sunnhemp (*Crotalaria juncea* L.) C.V Bellary were procured from the National Seed Corporation, Bangalore. After soaking overnight, seeds were surface sterilized as per the standard procedures with 1 % w/v bavistin and 0.1 % HgCl₂. The seeds were rinsed with sterile water in between treatment to remove traces of the sterilant. They were then allowed to germinate on sterile wet cotton in glass petriplates by incubating at 30 °C in dark for 24 h. Following germination the seed coat of the germlings were removed carefully using a sterile needle and were then used for infection.

Transformation The standardization conditions of transformation namely the survival rate of the germlings during co-cultivation, no of pricks per germling, amount of tobacco leaf extract to be used for induction of Vir genes etc. were determined empirically. These conditions were then employed in the procedure. The *Agrobacterium* host LBA4404 harboring the recombinant vector pCAMBIA 1D was grown over night in 25 ml of the L.B broth (pH 7.0, 50 µg ml⁻¹ kanamycin) at 28 °C. After a series of sub culturing for 2 days, the cells were pelleted and resuspended in 50 ml of Winnan's AB medium of pH 5.2 (Winans et al. 1988). After 12 h of growth in A.B medium, the *Vir* gene of *Agrobacterium* were induced by adding wounded tobacco leaf extract (Cheng et al. 1996) (2 ml for 50 ml of the AB medium). After 4 h of induction, the germlings which were wounded by gently pricking the cotyledonary node at the embryo axis 3–4 times using a sterile sewing needle were immersed in the A.B broth and incubated at 28 °C without agitation for one hour. Later, the germlings were treated with 500 µg ml⁻¹ of cefotaxime in liquid MS basal medium (Murashige and Skoog 1962) for 20 min (Rohini and Sankara Rao 2000) and then placed on moistened autoclaved soilrite (Keltec Industries, Bangalore) in 250 ml wide mouth bottles for further growth in the growth room maintained under standard conditions of 28 °C, and 14 h day time (30 µmol m⁻² s⁻¹). After 10–12 days the germlings were transferred to soilrite containing polythene bags and allowed to grow in the growth

Fig. 1 Schematic representation of recombinant vector pCAMBIA 1D

room. Later they were transferred to pots containing sand soil manure mix (1:2:1 ratio) and maintained in the greenhouse for growth flowering and seed set. The seeds of T_0 plants were collected and the next generation was raised. The experiment was repeated thrice with 75 germlings each time. The T_0 transformants showed no morphological changes when compared to the wild type plants.

Screening of transformants The genomic DNA from the fresh leaf tissue of the progeny plants in the green house was extracted individually by the CTAB method. (Doyle and Doyle 1987). The quality of DNA was analyzed by 0.8 % gel electrophoresis.

Genomic DNA PCR analysis The polymerase chain reaction was performed with the genomic DNA of the T_1 generation plants to determine the presence of the FMDV bivalent gene (1.3 kb) (ID of “O” and “A₂₂”) and also the marker gene nptII, (700 bp). For amplification of the FMDV gene, the reaction mix consisted of 10 ng (2 μ l) of the purified genomic DNA, 20 pm of each insert specific primer VP1 (O) Kp.Sp.Ml(L) 5' GCG GGT ACC GCA TGC GGA CGC GTG TAT GAC CAC CTC CCC GGG TGA G 3', and VP1(A22) EcoRI (R): 5' GCG AAT TCG CCG GGG TTC AAA AAC TTG CTT CTC AGG 3' 25 mM Tris HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 100 μ M each of the four dNTPs and 1 unit of Taq DNA polymerase (Bangalore Genei) in a total volume of 50 μ l. The mix was subjected to 35 cycles of amplification in thermal cycler after initial denaturation of the template at 94 °C for 3 min. Each cycle consisted of a denaturation phase of 1 min at 94 °C, followed by annealing at 56 °C for 1 min and primer extension at 72 °C for 1.5 min. The unfinished synthesis was allowed to complete in the final extension phase at 72 °C for 10 min. The presence of the marker gene nptII (700 bp) was detected by, hot start PCR with initial denaturation at 94 °C for 4 min followed by 35 cycles of 1 min at 94 °C, annealing for 1 min at 58 °C, primer extension [nptII (L): 5' GAG GCT ATT CGG CTA TGA CTG 3' nptII (R): 5' ATC GGG AGG GGC GAT ACC GTA 3' at 72 °C] for 1 min, followed by final extension phase at 72 °C for 10 min as described before. Both the amplicons were analyzed in 1 % agarose gel using the standard molecular weight markers.

Reverse transcriptase PCR The transcription of the transgene, bivalent 1D of “O” and ‘A₂₂’ (1.2 Kb) was analyzed by reverse transcriptase PCR. Total RNA from the genomic DNA PCR positive plants was extracted using the HiPurA™ Plant RNA Miniprep Purification Spin Kit. (Hi media, Bangalore). The cellular RNA was used to synthesize the cDNA as per the protocol recommended by the manufacturer's instructions (Superscript III, Invitrogen). The cDNAs were further amplified by PCR using the primers VP1 (O) Kp.Sp.Ml (L) 5' GCG GGT ACC GCA TGC GGA CGC GTG TAT GAC CAC CTC CCC GGG TGA G 3' and VP1 (O) Bam H1(R) 5' GCG GAT CCG CCG GGG GTT CAG GAG CTG CTT 3' with the same reaction conditions as mentioned above.

Analysis of the total protein of the transformants ELISA and Western blot of the total protein of the genomic DNA and the reverse transcriptase PCR positive plants was done to ascertain the expression and the antigenic nature of the expressed protein.

Total protein extraction from the plants The total protein of the plants positive for genomic DNA PCR and the reverse transcriptase PCR were extracted by the TCA-Acetone method (Damarval et al. 2007) with slight modifications. Briefly, the plant tissue (approx 2 g) was ground into fine powder with liquid nitrogen. The powdered tissue was then mixed with 1.5 ml of 10 % TCA- 0.07 % mercaptoethanol solution in cold acetone. This was kept on ice for one hour. The samples were then centrifuged at 12,000 g for 10 min at 4 °C and the pellet was rinsed with 50 μ l of 90 % cold aqueous acetone. After centrifugation the pellet was dried and dissolved in PBS (phosphate buffer saline pH 7.2). The total protein concentration of control and positive T_1 transgenic plants was estimated by the Bradford assay. (Bradford 1976).

FMDV protein detection by Western blot The total protein extracted from the plants was separated on 10 % SDS gel as per the standard procedure (Laemmli 1970). The protein bands from the gel were transferred on to a nitrocellulose (NC) membrane (Millipore) using TransBlot – SD electrophoretic transfer cell (BioRad) according to manufacturer's instructions. The membrane was then blocked for 1 h with

Fig. 2 Genomic DNA PCR of a few T_1 generation Sunnhemp plants using primers specific for FMDV-1D gene. Lane M: DNA marker; Lane 1–8: DNA of few transgenic plants; Lane N: DNA from non transformed plants; Lane P: pCAMBIA 1D plasmid DNA

5 % SMP (skimmed milk powder) in TBST (Tris buffer saline pH 7.6) with 0.045 ml Tween 20. The membrane was then washed with TBST and incubated with bivalent FMDV antibody raised in rabbit for 1 h at 37 °C. The membrane was washed again with TBST and incubated with goat antirabbit antibody conjugated with horse radish peroxidase (Sigma) for 1 h at 37 °C. The membrane was washed and developed with ODD (O-Dianisidine Dihydrochloride) substrate (Sigma).

Testing the antigenic nature of the transgenic plant protein samples by ELISA Initially the plant expressed antigen concentration to be coated was standardized. The plant extract were diluted in 1:5 ratios in coating buffer and coated on to the ELISA plates (Nunc Denmark) in duplicates at 4 °C overnight. The plates were washed thrice with PBST and saturated with 3 % SMP in PBS-T. The hyper immune sera raised in rabbit against the FMDV antigen 1D (1:2000) was added to the wells. The antigen antibody reaction was visualized by incubating with the goat anti rabbit antibody conjugated with horse radish peroxidase (Sigma) (1:1000) and chromogen OPD (O-Phenylenediamine, Sigma) as the substrate. The A_{492} nm was measured and the amount of the FMDV bivalent 1D protein expressed in each plant was determined from the standard curve prepared using the, *E. coli* expressed purified 1D protein as positive antigen.

Results and discussion

We have developed the *in planta* transformation protocol for Sunnhemp which is tissue culture independent based on the transformation reports of crops like *Arabidopsis thaliana* (Feldmann and Mark 1987), legumes like Soya bean (Olhoft and Somers 2001) and also on the reports that the cotyledonary nodes in Sunnhemp are the major sites of gene transfer (Hiroyuki daimon et al. 2002).

The major requirement for success of any *Agrobacterium* mediated transformation is the optimization of the experimental preconditions. The feasibility of the adapted transformation protocol was checked by performing some empirical experiments wherein the capacity of the germings to grow in to normal plants after wounding by pricking with a needle, the exposure time of the explants to the bacterium and also the amount of tobacco leaf extract to be used were determined. From our results, it was observed that more than 3–4 pricks using a very fine sewing needle will lead to permanent damage of the cotyledonary nodes and there will not be further growth of the germling. It was also evident that the exposure time of 45 to 60 min of the *Agrobacterium* to the explant is most feasible and more than one hour of exposure leads to a delay in the growth of the germings and also in some cases caused necrosis. Since previous studies suggested that the use of Acetosyringone did not improve the transformation efficiency (Rohini and Sankara Rao 2000), we used

Fig. 3 Genomic DNA PCR of a few T_1 generation Sunnhemp plants using primers specific for *npt II* gene. Lane M: DNA marker; Lane 1–4: DNA of few transgenic plants; Lane N: DNA from non transformed plants; Lane P: pCAMBIA 1D plasmid DNA

Fig. 4 Reverse transcriptase PCR of a few T_1 generation Sunnhemp plants using primers specific for 1D of FMDV serotype “O”. Lane M: DNA marker; Lane 1–4: RNA of few transgenic plants; Lane N: RNA from non transformed plants; Lane P: “O” virus RNA

wounded tobacco leaf extract (Rohini and Sankara Rao 2000) for virulence induction (2 ml of tobacco leaf extract for every 50 ml of Winnan’s medium).

The optimized conditions were repeated thrice successfully to generate the T_0 plants. Since we targeted the cotyledonary nodes which are highly meristematic for transformation, the resulting plants from this event will be chimeric and any kind of antibiotics selection process at this stage would have deleterious effects on the transformants. Hence, all the putative transformants were allowed to germinate on moistened soilrite and later transferred into pots to grow in green house. The growth of the transformed plants was slow when compared to the normal/control plants. The reason can be attributed to the stress induced on these plants due to pricking and exposure to *Agrobacterium*.

Since it has been reported that many legumes exhibit endogenous GUS like activity (Sudan et al. 2006), we chose to avoid the use of GUS gene as marker. Instead the FMDV ID gene of the two serotypes “O” and “A₂₂” which encodes for major antigenic protein and induces immune response in animals (Meloan and Barteling 1986) was linked together and its expression was used to assess the transformation efficiency.

Molecular analysis of the T_1 generation plants T_0 Plants took around 3 months (approx 90 days) to flower and set

seeds in the green house. They were phenotypically similar to the non transformed control plants. Out of 225 germlings transformed from the 3 separate experiments of 75 each, 60 plants were established. About 300 seeds were sown to raise the T_1 generation of which 180 plants were established. These were analyzed for integration of both bivalent FMDV gene 1D”O” and “A₂₂” and the nptII.

About 18 out of 180 T_1 generation plants showed amplification of the bivalent 1D gene (1.3 kb) (Fig. 2) as well as the nptII gene (700 bps) (Fig. 3) while the control plants did not show any amplification for both the genes. It was observed that 32 plants showed the presence of only nptII marker (fig not shown). This was in agreement with similar findings of presence of single marker in some plants by Yao et al. 1995 and Rohini and Sankara Rao 2000.

Based on the PCR results, the positive plants were further analyzed for the presence of mRNA transcript of the bivalent 1D gene by reverse transcriptase PCR (Fig. 4). Twelve out of 18 plants were found to contain the mRNA transcript. The absence of the transcript in other plants can be attributed to events like gene silencing or DNA methylation. It has been suggested by studies (Desai et al. 2010) that gene silencing and DNA methylations are positively correlated to copy number and expression of the transgene.

Fig. 5 western blot of T_1 generation Sunnhemp plants. Lane M: prestained protein marker; Lane 1, 2, 3, 4, 5, 6, 7: Total protein of transgenic plants; Lane N: negative control: Total protein from non transformed plant; Lane P: positive control: Protein expressed by BHK-21 cells transfected with the same gene constructs

Fig. 6 The amount of expressed bivalent ID “O” and “A₂₂” FMDV antigen/gm of the fresh leaves of the different independent transgenic lines

To confirm that the amplification is only due to the presence of RNA and not contaminated DNA, we conducted the direct PCR of the RNA samples. But none of the samples showed any amplification which confirmed that the amplification is only due to RNA and mRNA transcript present in the positive plants.

FMDV protein expression The integration of the marker bivalent 1D gene was confirmed by Western blot using hyper immune sera raised against the same antigen. Seven out of 12 transformants which tested positive for the presence of the bivalent gene ID (“O” and “A₂₂”) by genomic DNA and RT PCR, were randomly selected for the analysis of protein expression by Western blot with suitable controls. A single intense band of 48 kDa confirmed the expression of the bivalent 1D gene in all the seven plants (Fig. 5). The size of the expressed protein was in total agreement with the same protein expressed in the BHK –21 cells which was used as positive control.

The amount of bivalent FMDV antigen ID “O” and “A₂₂” expressed by each transgenic line was quantified by ELISA using the standard curve prepared with *E. coli* expressed bivalent FMDV antigen ID (“O” and “A₂₂”). The reaction of the expressed bivalent FMDV antigen 1D (“O” and “A₂₂”) with their specific antibodies suggests that the expressed protein is antigenically active. Since the integration of the gene in *Agrobacterium* mediated transformation is a random event (Jones et al. 1985), the expression levels also varied amongst the plants. The graph (Fig. 6) shows the amount of expressed antigen/gm of the fresh leaves of the different transgenic lines. Out of the 7 independent transgenic lines,

2 lines (transgenic lines 24 and 37) showed elevated levels of expression of 12 µg and 8 µg (per gm of fresh leaf) of the bivalent ID antigen “O” and “A₂₂” when compared to control plants suggesting that the copy number of the bivalent gene in these lines may be more than one. The expression may still be enhanced by using tissue specific promoters instead of constitutive promoters like CaMV 35S, since its expression is subjected to several regulatory elements and may reflect differential expression in different tissues of the same plants (Benfey and Chua 1990). Other modifications in the gene like codon optimization (Gustafsson et al. 2004), co-expression with protease inhibitors etc. may be tried for enhancing and stabilizing gene expression. The transformation efficiency in our experiments was found to be 3 %. (The results of the molecular analysis is summarized in Table 1) It is still preferred to use host strains like EHA105 with higher copy number of *Vir G* gene (Meurer et al. 1998) which may help in achieving increased efficiency.

This method of *in planta* transformation offers many advantages; it is less labor intensive and is reproducible. The transformants can be recovered by overcoming the problems of recalcitrancy to tissue culture conditions, generation of somaclonal variations etc. The method can also overcome genotype specificity barrier and thus, can be used for transformation of other Sunnhemp cultivars.

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Table 1 Summary of molecular analysis of the T₁ generation of Sunnhemp plants

No of germlings infected with LBA 4404 containing pCambia 1D	No of T ₀ plants established	No of seeds sown from T ₀ plants	No of T ₁ plants established	No of T ₁ plants testing positive:			Percentage of transformation efficiency
				Genomic DNA PCR of FMDV and nptII marker genes	reverse transcriptase PCR of FMDV genes	Western blot of FMDV genes	
225	60	300	180	18	12	7	3 %

References

- Benfey PN, Chua NH (1990) The cauliflower mosaic virus 35S promoter: combinatorial regulation of transcription in plants. *Science* 250(4983):959–966
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 7(72):248–54
- Carrillo C, Wigdorovitz A, Oliveros JC, Zamorano PI, Sadir AM, Gomez N, Salinas J, Escribano JM, Borca MV (1998) Protective immune response to foot and mouth disease virus with VP1 expressed in transgenic plants. *J Virol* 72:1688–1690
- Cheng M, Jarret RL, Li Z, Xing A, Demski JW (1996) Production of fertile transgenic peanut (*Arachis hypogaea* L.) plants using *Agrobacterium tumefaciens*. *Plant Cell Rep* 15:653–657
- Daimon H, Ohno H, Akasaka Y, Mii M (2002) A histological evaluation of adventitious bud formation in cotyledons in *Crotalaria juncea* L. *Plant Prod Sci* 5(4):301–304
- Damarval C, De Vienne D, Zivy M and Thiellement H (2007) Total Protein Extraction with TCA-Acetone. In: *Methods in Molecular Biology- Plant Proteomics: Methods and Protocols*. Humana Press Inc., Totowa, NJ
- Desai PN, Shrivastava N, Padh H (2010) Production of heterologous proteins in plants: strategies for optimal expression. *Biotechnol Adv* 28:427–435
- Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull* 19:11–15
- Dus Santos MJ, Wigdorovitz A, Trono K, Ríos RD, Franzone PM, Felix G, Moreno J, Carrillo C, Escribano JM, Borca MV (2002) A novel methodology to develop a foot and mouth disease virus (FMDV) peptide-based vaccine in transgenic plants. *Vaccine* 20:1141–1147
- Dus Santos MJ, Carrillo C, Ardila F, Ríos RD, Franzone P, Piccone ME, Wigdorovitz A, Borca MV (2005) Development of transgenic alfalfa plants containing the foot and mouth disease virus structural polyprotein gene P1 and its utilization as an experimental immunogen. *Vaccine* 23(15):1838–1843
- Feldmann KA, Mark MD (1987) *Agrobacterium*—mediated transformation of germinating seeds of *Arabidopsis thaliana*: a non tissue culture approach. *Mol Gen Genet* 208:1–9
- Gustafsson C, Govindarajan S, Minshull J (2004) Codon bias and heterogeneous protein expression. *Trends Biotechnol* 22(7):346–353
- Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. *Plant Mol Biol* 25:989–994
- Jones JDG, Dunsmuir D, Bedbrook J (1985) High level expression of introduced chimeric genes in regenerated transformed plants. *EMBO J* 10:2411–2418
- Laemmli UK (1970) Cleavage of structural protein during the analysis of head of bacteriophage T4. *Nature* 227:680
- Meloan RH, Barteling SJ (1986) An epitope located at C terminus of isolated VP1 of foot and mouth disease virus type “O” induces neutralizing activity but poor protection. *J Gen Virol* 67:289–294
- Meurer CA et al (1998) Factors affecting soya bean cotyledonary node transformation. *Plant Cell Rep* 18:180–186
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15: 473–497
- Ogra PL, Faden H, Welliver RC (2001) Vaccination strategies for mucosal immune responses. *Clin microbial rev* 14:430–445
- Ohara A, Akasaka Y, Daimon H, Mii M (2000) Plant regeneration from hairy roots induced by infection with *Agrobacterium rhizogenes* in *Crotalaria juncea* L. *Plant Cell Rep* 19(6):563–568. doi:10.1007/s002990050774
- Olhoft PM, Somers DA (2001) L-Cysteine increases *Agrobacterium*-mediated T-DNA delivery into soybean cotyledonary-node cells. *Plant Cell Rep* 20:706–711
- Olhoft PM, Lin K, Galbraith J, Nielsen NC, Somers DA (2001) Role of thiol compounds in increasing *Agrobacterium* mediated transformation of Soya bean cotyledonary nodes cells. *Plant Cell Rep* 20:731–737
- Pandey VN, Srivastava AK (1990) Seed protein yield from some *Crotalaria* spp and *in vitro* nutritional quality of that from *C. juncea*. *Plant foods Human Nutr* 40:195–200
- Prasad V, Satyavathi VV, Sanjaya, Valli KM, Abha khandelwal, Shaila M S, Lakshmi sita G (2004) Expression of biologically active Hemagglutinin-Neuraminidase protein of *Peste des petits ruminants* virus in transgenic pigeon pea [*Cajanus cajan* (L) Millsp]
- Purseglove JW (1981) Leguminosae. In: Purseglove JW (ed) *Tropical crops: Dicotyledons*. Longman group ltd, Essex, pp 250–254
- Rohini VK, Sankara Rao K (2000) Transformation of peanut (*Arachis hypogaea* L.): a non-tissue culture based approach for generating transgenic plants. *Plant Sci* 150:41–49
- Sudan C, Praksh S, Bhomkar P, Jain S, Bhalla-Sarin N (2006) Ubiquitous presence of β -glucuronidase (GUS) in plants and its regulation in some model plants. *Planta* 224(4):853–864
- Trieu AT, Burleigh SH, Kardailsky IV, Maldonado-mendoza IE, Versaw WK, Blaylock LA, Shin H, Chiou T-J, Katagi H, Dewbrew GR et al (2000) Transformation of *Medicago truncatula* via infiltration of germlings of flowering plants with *Agrobacterium*. *Plant J* 22:531–541
- Wen-Jun S, Forde BG (1989) Efficient transformation of *Agrobacterium* spp. by high voltage electroporation. *Nucleic Acid Res* 17:8385
- Winans SC, Kerstetter RA, Nester EW (1988) Transcriptional regulation of the *Vir A* and *Vir G* genes of *Agrobacterium tumefaciens*. *J Bacteriol* 170:4047–4054
- Yao JL, Cohen D, Atkinson R, Richardson K, Morris B (1995) Regeneration of transgenic plants from the commercial apple cultivar Royal gala. *Plant Cell Rep* 14:407–412