Asian Journal of Plant Sciences

ISSN 1682-3974

science alert

ANSInet
an open access publisher
http://ansinet.com

Asian Journal of Plant Sciences 12 (3): 97-108, 2013
ISSN 1682-3974 / DOI: 10.3923/ajps.2013.97.108

Agrobacterium-mediated Transformation of Pearl Millet (*Pennisetum typhoides* (L.) R.Br.) for Fungal Resistance

[1]S. Ignacimuthu and [2]P. Kannan
[1]Division of Plant Biotechnology, Entomology Research Institute, Loyola College, Chennai, 600 034, India
[2]Department of Botany, The Madura College, Madurai, 11, India

Abstract: A new *Agrobacterium*-mediated transformation system was developed for pearl millet using shoot apex explants, conferring resistance to leaf blast disease by inserting a rice chitinase (chi11) gene. Transgenic pearl millet lines (*Pennisetum typhoides* (L.) R.Br.) expressing rice chitinase gene with high levels of resistance to rust pathogen, *Puccinia penniseti*, were developed using *Agrobacterium*-mediated gene transfer method. The emryogenic calli derived from shoot apex of CO9 cultivar were transformed with LBA4404 (pSB1/pKAN-Rchit1.1) that harboured rice chitinase gene (chit11) under the control of maize ubiquitin (Ubi 1) promoter intron. Transgene (chit 11) in the middle of the T-DNA as used as probe in southern analysis. Out of six independent T_0 plants tested for southern, three had single copy T-DNA insertions and three had two copies T-DNA insertions. All the six T_0 plants carried complete T-DNA with the chitinase transgene. A segregation ratio of 3:1, reflecting T-DNA insertion at a single locus, was observed in the progeny of all the T_0 plants which showed normal Mendelian pattern of transgene segregation. Western blot analysis of T_1 plants revealed constitutive expression of chitinase at high levels. Bioassays of T_1 plants indicated enhanced resistance to the rust pathogen, *P. penniseti*, in comparison to control plants. This is the first report on *Agrobacterium*-mediated transformation of pearl millet and first transgenic pearl millet with fungal resistance. This study underpins the introduction of numerous agronomically important genes into the genome of pearl millet in the future.

Key words: *Agrobacterium*, pearl millet, shoot apex, rice chitinase gene, transgenic plants, fungal resistance

INTRODUCTION

Pearl millet (*Pennisetum typhoides* (L.) R.Br.) is one of the major cereal crops worldwide (Girgi *et al*., 2006). It is a high yielding summer crop tolerant to drought and acidity. It can be grown in low rainfall areas where maize and sorghum do not thrive (FAO, 2004). It occupies 40 million ha in the drought-prone semi-arid and arid tropics of Asia and Africa. It meets 80-90% of the calorie necessities of millions of people inhabiting these regions (Lambe *et al*., 2000; O'Kennedy *et al*., 2004a). In the past one decade the net productivity of pearl millet has been limited because of various reasons such as fungal diseases, insect pests, drought stress, high soil temperature and inferior agronomic characteristics (Rai *et al*., 1997; Gueye and Delobel, 1999; Grover and Pental, 2003; Ceasar and Ignacimuthu, 2009). The most important yield constraints of pearl millets are fungal diseases contributing to yield losses (Grover and Pental, 2003; Latha *et al*., 2006; Girgi *et al*., 2006). Pearl millet is susceptible to several fungal diseases such as rust disease caused by basidomycete *Puccinia penniseti*, Downey mildew caused by the *Oomycete sclerospora graminicola* and smut caused by *Ustilago* sp. (Wilson, 2000; Grover and Pental, 2003). Among the different fungal pathogens *P. penniseti* is one of the most damaging fungi in many countries (Singh *et al*., 1993; Girgi *et al*., 2006). Breeding for resistance to above mentioned diseases is a high priority for pearl millet breeders (Anonymous, 1996).

Traditional breeding has been the main opportunity for crop development in pearl millet. A number of approaches were taken to improve fungal resistance in plants such as expression of Pathogenesis Related (PR) proteins (Zhu *et al*., 1994; Van Loon and van Strien, 1999; Kishimoto *et al*., 2002) and phytoalexins (Hain *et al*., 1993; Dixon, 2001). Gene transfer has become an established and routine technique in many laboratories. Genetic transformation of pearl millet with antifungal genes would help in the management of various fungal pathogens (Sharma and Ortiz, 2000; Thakur and Mathur, 2002; Devi *et al*., 2000; Girgi *et al*., 2006; Ceasar and Ignacimuthu, 2011).

Taylor and Vasil (1991) and Taylor *et al*. (1993), using microprojectile bombardment, incorporated gus A gene into the scutellum of immature embryos of pearl millet and

Corresponding Author: S. Ignacimuthu, Division of Plant Biotechnology, Entomology Research Institute, Loyola College, Chennai, 600 034, India Fax: 91-44-2817 4644

reported GUS expression. Lambe *et al.* (1995) transferred gus A, hpt, nptII and bar genes into pearl millet through microprojectile method. Girgi *et al.* (2002) transferred gus A and bar genes into the scutellar tissue of immature embryos. Devi and Sticklen (2002) described a rapid, reliable method for the microprojectile bombardment and transient expression of GUS in the multiple-shoot tip clumps of pearl millet. Transgenic pearl millet plants harbouring bar and gfp genes were also developed using Particle Inflow Gun (PIG) (Goldman *et al.*, 2003). A transformation protocol was established with the herbicide resistance selectable marker gene, bar, using PIG (Girgi *et al.*, 2002). The manA selection system was used as selectable markers in pearl millet transformation (O'Kennedy *et al.*, 2004b). Recently, using precultured immature zygotic embryos and embryogenic tissue of maize and pearl millet, transformation was developed by biolistic method (O'Kennedy *et al.*, 2011).

Latha *et al.* (2006) reported that a reproducible method for genetic transformation employing gus A gene with PIG method in pearl millet. A chemically synthesized antifungal pin gene was used for producing transgenic pearl millet (ICMP451) with resistance to downey mildew *S. graminicola*. Transgenic pearl millet was developed against rust (*P. substriata*) and downey mildew (*S. graminicola*) by introducing a cDNA encoding the antifungal protein AFP from the mould *Aspergillus giganteus* (Girgi *et al.*, 2006). To the best of knowledge, there are no reports on *Agrobacterium*-mediated gene transformation in pearl millet so far. Further transgenic pearl millet with rice chitinase gene resistant to rust disease has not yet been developed. Hence, the present study was aimed at using *Agrobacterium*-mediated transformation system to stably introduce chitinase gene into pearl millet for developing rust disease resistance.

MATERIALS AND METHODS

Plant materials and *Agrobacterium* strain: Pearl millet (*Pennisetum typhoides* (L.) R.Br.) cultivar CO9 was obtained from Tamil Nadu Agricultural University, Coimbatore, India. Mature, healthy seeds were surface sterilized with 70% alcohol for 30 sec and in 0.1% $HgCl_2$ (w/v) for 5 min, followed by five rinses with sterile double distilled water. Sterilized seeds were cultured on MS basal medium (Murashige and Skoog, 1962) with 3% sucrose for germination. Three-day-old shoot apices (2-3 mm) removed from the seedlings were used for transformation experiments. *Agrobacterium tumefaciens* strain EHA 105 harboring LBA4404 (pSB1/pKAN-Rchit1.1) was used for transformation. A 3.1kb Hind III fragment obtained from pCAMBAR CH11 (kindly provided by Dr. Muhtukrishnan, Kansas State University, USA) containing rice chitinase gene, ubiqutin promoter and Ubi1 was subcloned into a binary vector LBA 4404 (pSB1), Kan^r (Fig. 1). T- DNA of the vector carried highly

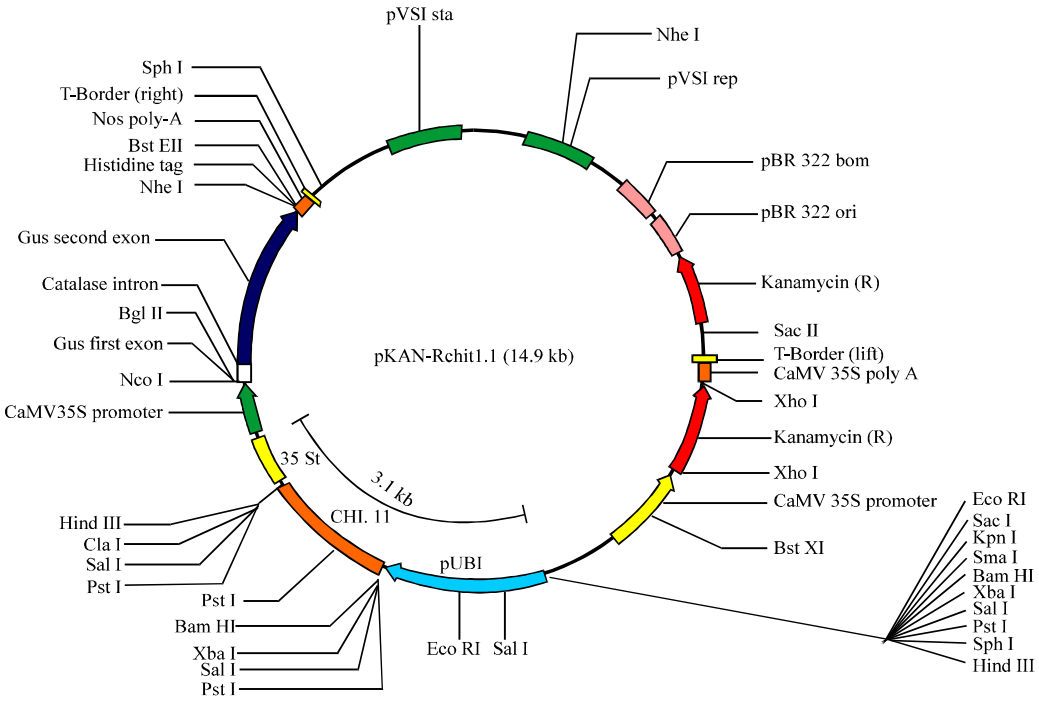

Fig. 1: Plasmid construct LBA 4404 (pSB1/pKAN-Rchit 1.1), showing restriction sites. LB, RB Left and right borders, respectively

active maize ubiqutin promoter with its intron 1 fused with rice chitinase gene driven by CaMV 35S promoter and nos polyA terminator. The plasmid contained GUS as reporter gene and kanamycin neophosphotransferase (npt II) as selection marker.

***Agrobacterium* infection and cocultivation:** *A. tumefaciens* strain was grown for three days on AB minimal, medium (Chilton *et al*., 1974) with 50 mg L^{-1} kanamycin 10 mg L^{-1} rifamycin and 10 mg L^{-1} tetracycline. A single colony was transferred to AB minimal liquid medium containing the above mentioned antibiotics and the culture was allowed to shake overnight at 28°C at 200 rpm. The overnight culture (0.5 mL) was transferred to 50 mL of fresh AB medium containing the selective antibiotics. The cultures were grown over night under the same conditions. When the Optical Density (OD) of *Agrobacterium* reached 0.6 (600 nm), the bacterial culture was centrifuged at 5000 rpm for 10 min at 4°C and pelleted. It was resuspended in equal volume of AA medium (Toriyama and Hinata, 1985) to obtain a density of 10^8 cells mL^{-1} which contained 30 g L^{-1} sucrose and acetosyringone (AS) 100 μM, pH 5.6 (AA-AS). The culture was allowed to shake again for 3 h under the same conditions as described above and this *Agrobacterium* culture was used for infection of the shoot apex calli explants.

The shoot apex calli (0.5-1.0 cm) were immersed in the bacterial suspension for 30 min with occasional shaking in the shaker at 150 rpm. Excess bacteria were removed from the surface of explants by placing them on sterile Whatman No. 1 filter paper before transfer to semisolid (0.6% agar) cocultivation medium consisting of 2, 4-D 2.0 mg L^{-1} and BAP 0.5 mg L^{-1} with 100 μM AS (PM-CCM). The effect of cocultivation period was evaluated and the shoot apex calli were assayed for transient GUS activity. After cocultivation period, the explants were removed from the cocultivation media and rinsed 4-5 times with sterile distilled water containing 250 mg L^{-1} cefotaxime and 300 mg L^{-1} carbenicillin to eliminate *Agrobacterium*.

Selection and regeneration of transgenic pearl millet: The inoculated calli were transferred to MS medium supplemented with 2,4-D 2.0 mg L^{-1}+BAP 0.5 mg L^{-1}+ 120 mg L^{-1} kanamycin+300 mg L^{-1} carbenicillin (PM-SL1) for inhibition of bacterial growth and selection of transformed tissues. The kanamycin resistant shoot apex calli were transferred to fresh selection medium consisting of 2, 4-D 2.0 mg L^{-1}+BAP 0.5 mg L^{-1}+120 mg L^{-1} kanamycin+300 mg L^{-1} carbenicillin (PM-SL2). After three rounds of selection and total of 6 weeks on selection medium the calli were transferred to regeneration medium fortified with BAP 2.0 mg L^{-1}+NAA 0.5 mg L^{-1}+ 120 mg L^{-1} kanamycin+300 mg L^{-1} carbenicillin (PM-RE). Small pieces of the kanamycin resistant calli as well as their respective untransformed control calli were assayed for GUS activity every three weeks during subculture. The kanamycin resistant regenerated shoots (about 3-5 cm length) were transferred to MS medium containing BAP 2.0 mg L^{-1}+NAA 0.5 mg L^{-1}+120 mg L^{-1} kanamycin+ 300 mg L^{-1} carbenicillin for shoot development. The regenerated shoots were further transferred to MS medium containing 0.5 mg L^{-1} IBA+120 mg L^{-1} kanamycin+300 mg L^{-1} carbenicillin for rooting (PM-RIM). Two weeks old rooted plantlets (8-10 cm length) were individually transferred to plastic cups (10×50 cm) containing sterile garden soil and vermiculite at 1:1 ratio and watered with sterile half strength Hoagland's solution (Hoagland and Arnon, 1950). After covering the cups with polythene bag, the set up was maintained for 7-10 days in the culture room at 27±2°C under a photoperiod regime having 16 h light. Hardened plants were transferred to earthen pots containing garden soil and maintained in the green house.

Histochemical assay for the GUS gene: The expression of β-D-Glucuronidase (GUS) gene in shoot apex calli, leaves and roots were assayed with 5-bromo-4-chloro-3-indolyl glucuronide (X-Glu) substrate essentially following the method of Jefferson (1987). The pearl millet tissues were incubated in sodium phosphate buffer (50 mM $NaPO_4$, pH 6.8) that contained 1% Triton X- 100 at 37°C for 1 h. Fresh phosphate buffer containing 1.0 mM X-Gluc and 20% methanol replaced the buffer. The reaction mixture was incubated overnight at 37°C and then tissues were examined visually for dark blue sectors.

PCR analysis of putative transgenic plants: Total genomic DNA was extracted from young leaf tissues of T0 GUS positive and untransformed control plants (Roger and Bendich 1994). Genomic DNA of putative transformants was subjected to PCR analysis with chit11 and npt-II gene primers. Polymerase Chain Reaction (PCR) was carried out in a 25 μL reaction mixture containing template DNA (50 ng), 120 μM of each dNTPs, 2 pmol of specific primers for npt-II gene (Forward (5' GCCATTTGAAGCCGATGTCAC 3') and reverse primer (5'-TCTGCCCCAACTGCCTCTGCT-3'), chit11 (Forward (5'-CCCCGCGGCCGTAGTTGTAGT-3') and reverse primer (5' AGAGAGGTTAAAGGCCGACAGC 3'), 1 unit Taq DNA polymerase, 3 mM $MgCl_2$ and 1x Taq DNA polymerase buffer. The reaction was carried out at 94°C for 1 min, followed by 30 cycles of 94°C for 1 min, 55°C for

1 min and 72°C for 1 min. This was followed by one cycle of 10 min at 72°C. The reactions were carried out in an eppendorf thermocycler. The amplified products were assayed by electrophoresis on 0.8% agarose gels, stained with ethidium bromide (EtBr; 0.5 g mL^{-1}), visualized and photographed under ultraviolet light.

Southern hybridization: Southern analysis was performed as described by Southern (1975). The genomic DNA of transformed and control plants were digested with Hind III restriction enzyme to express the 3.1 kb chitinase gene and electrophoresed in 1% agarose gel to get the band. The DNA bands were denatured and transferred to a nylon membrane by standard method. Southern hybridization was carried out using radioactive labeling method. The probe DNA of GUS gene was labeled [α-^{32}P]dCTP (BRIT, Mumbai, India) using a random primer labeling kit (Amersham International, Plc. Ltd., London, UK). To confirm the presence of chitinase gene in transgenic plants, 1.1 kb chit11 coding sequence was used as probe. Hybridization was carried out at 65°C. Post-hybridization washes were done sequentially with 3xSSC (1xSSC is 0.15 M NaCl plus 0.015 M trisodium citrate), 0.5xSSC and 0.1xSSC, along with 0.1% Sodium Dodecyl Sulfate (SDS); each wash was carried out for 30 min at 65°C. After hybridization washes, the membrane was exposed to X-ray film and incubated for 48 h at -80°C.

Western blot analysis: Western blot analysis was performed to detect the expression of chitinase enzyme in T_0, T_1 progenies and untransformed plants. Protein extraction and western analysis were performed as described by Chen *et al.* (1998). Total protein was isolated from 1 g young leaves of 35-day-old of T_0 progeny, T_1 progeny and untransformed plants were ground to a fine powder using liquid nitrogen and homogenized with extraction buffer (Bradford, 1976) supplemented with 10 mM β-Mercabtoethanol. The extract was centrifuged at 18,000 x g for 20 min at 4°C and the supernatant was used for western analysis. The protein concentration was estimated using the method of Bradford (1976). Twenty microgram aliquots of total protein were separated by SDS-PAGE in a 10% gel and transformed to a nitrocellulose membrane using a semi-dry transfer apparatus. Molecular weight markers (Rainbow marker) were purchased from Amersham Pharmacia Biotech, Little Chalfot, England. The membrane was blocked using 3% gelatin and Tween-Tris Buffered Saline (TTBS) and probed with the chitinase antibody (a polyclonal rabbit antibody raised against barley chitinase kindly provided by Dr. S. Muthukrishanan, Kansas State University) diluted to 1:1000 (v/v). The second antibody, goat anti-rabbit IgG (H+L) alkaline phosphates conjugate from Bangalore Genei Pvt. Ltd., Bangalore, India was used at a dilution of 1:2,000. The membrane was treated with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) colour reagent until bands appeared.

Chitinase enzyme assay: Chitinase enzyme assay was performed to confirm the level of chitinase enzyme produced in kanamycin resistant plants and compared with control plants. The colorimetric assay of chitinase was carried out following the method of (Mauch *et al.*, 1988; Ceasar and Ignacimuthu, 2012) using colloidal chitin as the substrate.

Leaf blast assay: Fungal bioassay was carried out on T1 plants (2T1, 5T1 and 9T1) and on untransformed seedlings under controlled conditions in the green house. *Puccinia* spores were obtained from Division of Plant Pathology, Tamilnadu Agricultural University, Madurai, India. Spores of *Puccinia* were suspended in sterile distilled water and the spore suspension (10^6 spores mL^{-1}) was sprayed on 10-days old seedlings maintained at 20-25°C and 85-90% relative humidity. Symptoms of the disease were recorded 10-days after spore inoculation and disease intensity was graded on 0-9 scale, where 0 indicated maximum resistance and 9 indicated maximum susceptibility resulting in seedling death. The size of the leasions was measured using the scale provided in the microscope. Plants falling in the range of 0-4 scale were classified as resistant and seedlings in 5-9 scale were scored as susceptible. The experiments were carried out in triplicate and repeated twice. The Chi-square test for goodness of fit was also applied to confirm the inheritance of rice chitinase gene in plants grown to maturity under controlled conditions in the greenhouse.

Segregation analysis of transgene in T_1 progenies: A segregation analysis was performed to check the pattern of inheritance. Seeds collected from selfed T_0 plants were screened for kanamycin resistance; the seeds were germinated initially on MS basal medium containing 3% sucrose (solidified using 0.8% agar) and placed in dark. The germinated seedlings were then transferred to the same medium supplemented with 120 mg L^{-1} kanamycin and placed in light. After 8 days, seedlings were scored for kanamycin resistance (kanS and kanR) and the data were validated using χ^2 test. Root and leaf segments were used for GUS assay. From this, segregation was analyzed.

Data analysis: The entire experiments were carried out in an absolutely randomized design. All experiments were replicated three times, each replicate consisting of more

than ten explants based on the research. The mean frequency (%) of transient GUS appearance (number of explants producing blue spots/total number of explants×100) was calculated 3 weeks after infection and mean frequency (%) of constant transformation (number of transgenic plants regenerated/total number of explants×100) was calculated after 7 weeks under regeneration on kanamycin. Data were analysed statistically (ANOVA or χ^2 test) and the mean and standard deviation were calculated for each experiment. The Fisher's Least Significant Difference (LSD) (Fisher, 1935) was calculated at 5% level with the statistical package for social science (Version 12.0 for Windows, SPSS Inc.).

RESULTS

Pearl millet transformation and regeneration: The gene for npt-II encoding kanamycin phosphotransferase confers resistance to kanamycin. In order to determine the usefulness of kanamycin resistant gene (npt) for the selection of transformed pearl millet tissues, killing curve was established for pearl millet shoot apex calli with different concentrations of kanamycin. The selection media contained MS basal medium supplemented with 2 mg L^{-1} of 2, 4-D and 0.5 mg L^{-1} BAP (PM-SL1). The cultures were incubated for one month. It was seen that no shoot apices survived on 120 mg L^{-1} kanamycin. Therefore, 120 mg L^{-1} was chosen for selection of transformants throughout the transformation experiments. Higher concentration of kanamycin induced necrosis of the explants very rapidly and reduced the survival rate of the explants.

The chitinase gene was introduced into shoot apex derived calli of the pearl millet cultivar CO9 by *Agrobacterium* mediated gene transfer method. A total of three experiments were carried out to determine the transformation efficiency. Shoot tip calli cocultivated with LBA 4404 (pSB1/pKAN-Rchit 1.1) for 3 days produced kanamycin resistant calli after 35 days. The selection medium (PM-SL2) containing 120 mg L^{-1} kanamycin and 300 mg L^{-1} carbenicillin (Fig. 2a) was used. Once in 21 days the calli were subcultured for vigorous selection. Growth of the calli which were not infected with *Agrobacterium* LBA 4404 (pSB1/pKAN-Rchit 1.1) was efficiently inhibited in a medium containing 120 mg L^{-1} kanamycin (negative control). The positive control calli (not infected with *Agrobacterium*) efficiently proliferated in the callus induction medium in the absence of kanamycin. A high frequency of kanamycin resistant calli was observed in three different experiments. Out of 961 calli cocultivated in three experiments 437 kanamycin resistant calli were obtained; most of the kanamycin resistant calli exhibited blue staining for GUS activity in the histochemical GUS assay (Table 1).

After 63 days of selection the kanamycin resistant calli were transferred to regeneration medium containing 120 mg L^{-1} kanamycin and 300 mg L^{-1} carbenicillin (PM-RE). Small shoot clusters (Fig. 2b) were observed on the transformed calli after three weeks of growth and the regenerating calli were subcultured in fresh regeneration medium and maintained for another three weeks. In three experiments, a total of 27 kanamycin resistant plants were regenerated from 60 calli. After four weeks in regeneration medium the plantlets rooted (Fig. 2c-d) on the rooting medium (PM-RIM). Small leaf pieces of various parts of the putatively transformed shoots were used for GUS histochemical assay. In these assays, 25 of the 71 regenerated shoots showed blue precipitate (Fig. 2e). These plantlets were hardened (Fig. 2f) and transferred to field conditions to set seeds. This cultivar CO9 showed a transformation frequency ranging from 6.0- 8.7% (Table 1).

PCR and southern analysis of transgenic plants: PCR was carried out on the genomic DNA isolated from the T_0 tranformants and the untransformed control using primers of npt-II, GUS and chitinase coding sequences. All the transformants were found to be positive for the amplification of 0.7 kb (npt II) and 1.1 kb (chitinase) genes by PCR. In all transformed plants the npt II fragment (0.7 kb) was amplified; only a faint band was observed in plants (lanes) 4, 11 and 12 while no such band was amplified in the untransformed control (Fig. 3a). Of the six plants used to check the chitinase (chit11) transgene fragment (1.1 kb), all the plants showed the expected fragment size (Fig. 3b). A similar band was also noticed in the positive plasmid control while no such band was observed in the untransformed control. This indicated that the tissues were completely free of *Agrobacterium*.

Table 1: Transformation efficiency (TF) by *Agrobacterium* LBA4404 (pSB1/pKAN- Rchit1.1) with rice chitinase in shoot apex calli of pearl millet cultivars, CO9

Pearl millet cultivars	Experiment No.	No. of shoot apex calli cocultivated (A)	Explants produced kan^R plant lines	kan^R and GUS+plant lines	No. of plants positive for PCR and southern (B)	Transformation efficiency (%) (B/A)
CO9	1	308	118	32	27	8.7
	2	340	38	17	27	7.9
	3	313	81	11	19	6.0

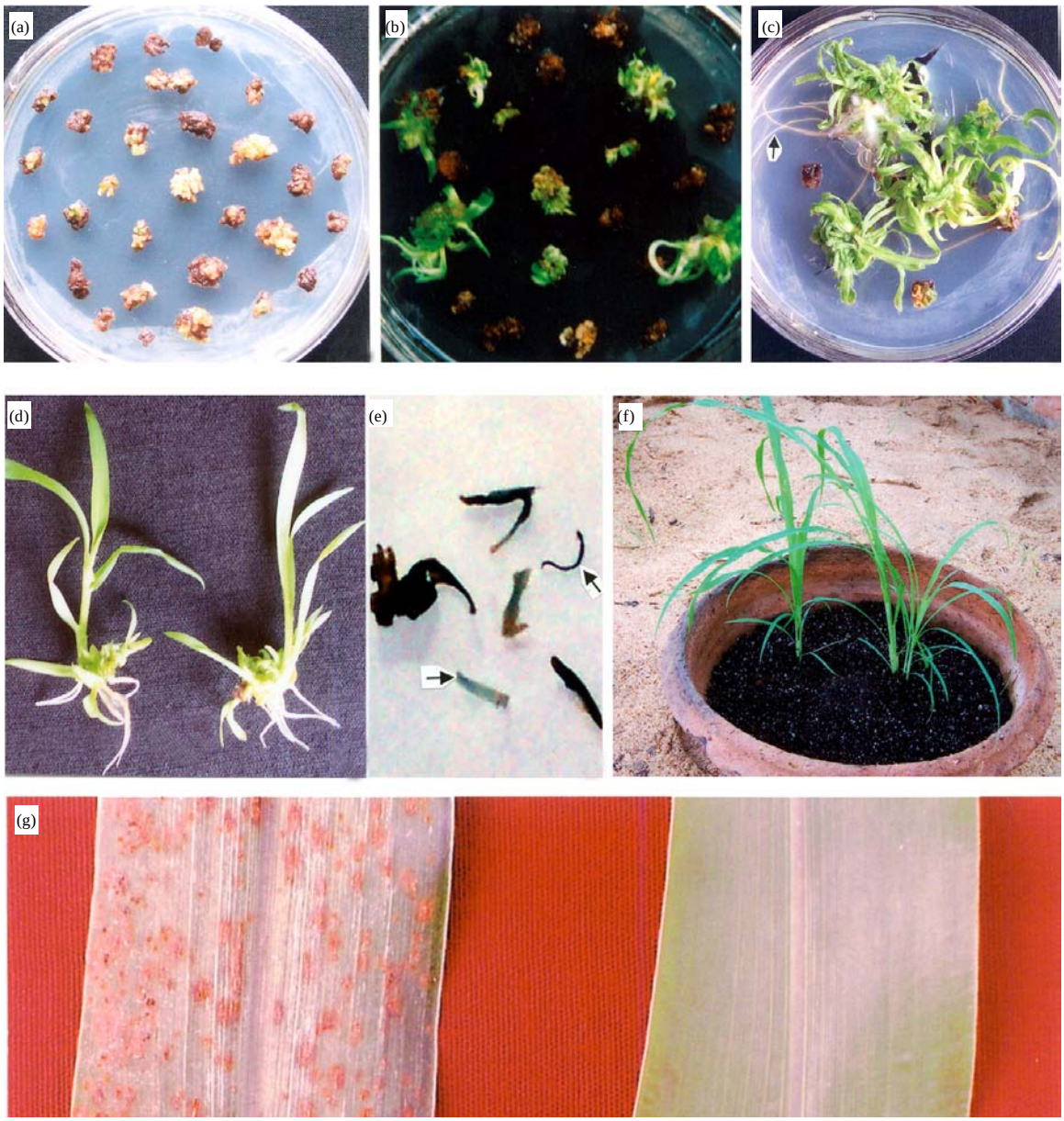

Fig. 2(a-g): *Agrobacterium*-mediated transformation of pearl millet (*Pennisetum typhoides* (L.) R.Br.cv. CO9) (a) Transient expression of GUS gene in shoot apex derived calli of pearl millet after cocultivation with *A. tumefaciens* for 3 days (scale bar = 3 mm), (b) Pearl millet plant regeneration from shoot apex calli explants after 21 days, (c) Root induction and whole plant formation in selection (regeneration) medium, black arrow showing root formation, (d) A transformed pearl millet plants recovered from selection medium, (e) GUS expression in explants of pearl millet transformed with rice chitinase gene (chi11) using *A. tumefaciens* LBA4404 (pSB1/pKAN-Rchit 1.1), black arrow showing blue colour, (f) Transformed plants in green house condition, (g) Leaf adaxial surface of the transgenic plants (right) not showing fungal infection, susceptible untransformed control (left)`

Southern blot analysis was performed in order to prove stable integration of chitinase gene within plant genome. DNA from six of the GUS positive transgenic shoots was used for molecular analysis. Total DNA (20 μg) of putative transformants was digested with Hind III restriction enzyme which released an internal fragment of expected size of 3.1kb rice chitinase gene along with npt-II and GUS reporter. The probe for chitinase gene was prepared from pCAMBAR CHI 1.1 plasmid by digesting with sac I restriction enzyme and it was radioactive labeled. Chitinase gene probe hybridized only to DNA from transgenic plants (Fig. 4a) but not to the DNA from the untransformed control plants. The result indicated that chitinase gene was integrated in the pearl millet genome.

Segregation analysis of transgene: Selfed progeny of four independent transformants of CO9 cultivar

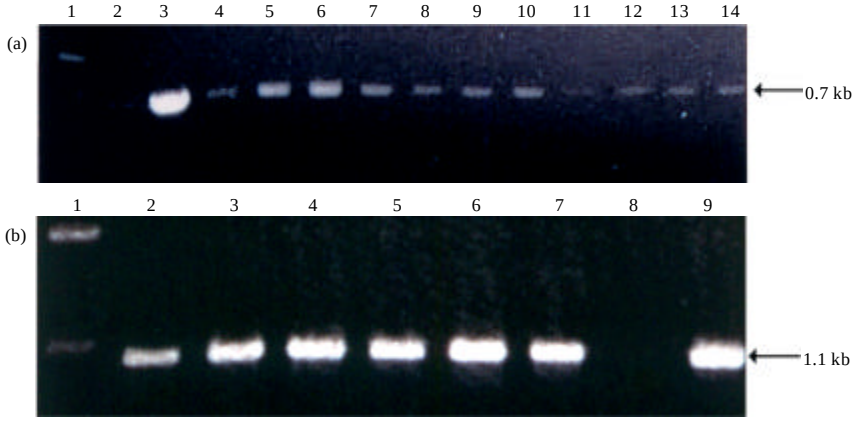

Fig. 3(a-b): (a) PCR analysis of transgenic plants with 0.7 kb fragment of npt-II gene. Lane 1: 1 kb Marker ladder, Lane 2: Untransformed plant as negative control, Lane 3: pCAMBIA2301 plasmid as positive control and Lanes 4-14: Putative transgenic plants of CO9, (b) PCR analysis of DNA of transgenic plants with 1.1 kb fragment within the chit 11 gene, Lane 1: One killobyte ladder, Lanes 2-7: Putative transgenic plants of CO9, Lane 8: Untransformed plant as negative control and Lane 9: pCAMBIA 2301 with chit 11 plasmid as a positive control

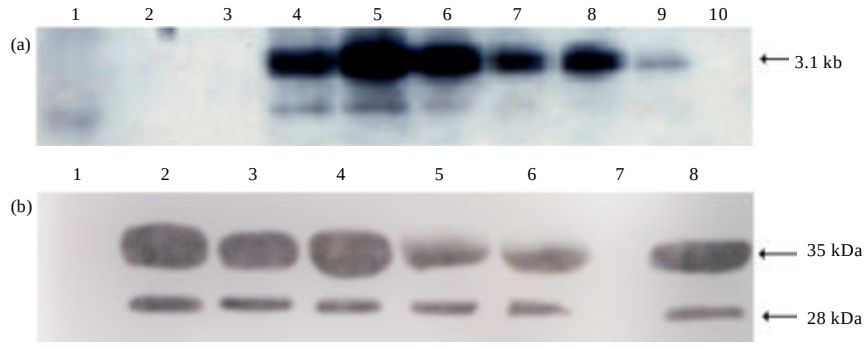

Fig. 4(a-b): (a) Southern blot analysis of T_0 transgenic plants. A chit 11 gene fragment was used to probe genomic DNA isolated from leaves of transgenic and untransformed lines. The blot was digested with Hind III and loaded in each lane. They were hybridized with radiolabeled dCTP- chit 11 (3.1 kb) coding sequences, Lane 1: λ Hind III digested marker, Lane 2: Undigested DNA from T_0 plants, Lane 3: Untransformed control, Lanes 4-9: Transformed plants and Lane 10: One killobyte Marker, (b) Western blot analysis of T_1 plants of three T_0 lines for chitinase expression. Twenty microgram aliquot of total protein was loaded in each lane and separated by SDS-PAGE in 10% polyacerylamide gels, Lane 1: Rainbow marker, Lanes 2-6 and 8: Transgenic plants of CO9 and Lane 7: Untransformed plants

Table 2: Segregation of kanamycin resistance gene in T_1 generation of pearl millet plants transformed with *Agrobacterium*

		Kanamycin				GUS			
Transformation No.	Total No. of seeds tested	Resistant	Sensitive	Ratio	χ^2 value	Positive	Negative	Ratio	χ^2 value
CO9-2	49	31	11	3:1	0.10	31	11	3:1	0.10
CO9-4	25	20	5	3:1	0.82	25	5	3:1	0.82
CO9-8	44	34	14	3:1	0.07	24	24	1:1	0.07

transformed with LBA4404 (pSB1/pKAN-Rchit 1.1), were examined for npt-II gene resistance. Resistant and sensitive seedlings were distinguishable on MS basal medium containing 120 mg L^{-1} kanamycin (PM-GM). These sensitive plants died within two weeks after the treatment while the resistant plants were as healthy as untreated plants. Further GUS expression was also observed in the corresponding plants. The sensitive plants died after the treatment while the resistant plants were alive. All the four transgenic lines exhibited a segregation ratio of 3:1 (Table 2).

Western blot analysis of putative transgenic pearl millets: Western blot analysis was performed with six T_1 transgenic plants. Total soluble protein was extracted from the leaf tissues of control and transgenic plants. In each lane 20 μg of total protein was loaded for SDS-PAGE analysis. Barley chitinase antibody served as the primary antibody. The results showed high accumulation of 35 kD chitinase in transgenic plants. No signal was seen in control plants. The size of the protein matched the expected relative mass of 35,000 for chitinase (Fig. 4b) in addition to the 35 kD protein; a band at 28 kD was also detected in all six T_1 plants. The 28 kD protein may have been released by proteolysis. Comparable levels of chitinase were accumulated in all the six transgenic lines. There was slight difference in the levels of chitinase in transformed plants presumably because of the degree of the expression of chitinase gene.

Bioassay of transgenic pearl millet plants with rust pathogen *P. penniseti*: Fungal bioassay on T_1 progenies of three lines of T_0 transformants was done to assess the antifungal activity of chitinase gene against the fungal pathogen *P. penniseti*. The symptom was observed for 10 days after fungal infection. The size of *Puccinia* pustules on control untransformed plants was bigger at 15 days post inoculation on the leaves. Rust infection of the leaf segments from the transformed pearl millet plants was significantly reduced. The trangenics showed significant disease resistance based on the degree of symptoms. The untransformed control seedlings were very much susceptible to fungal spores showing severe disease symptoms (Fig. 2g). Bioassay on T_1 progenies of T_0 transformants showed monogenic segregation of 3 resistant: 1 susceptible plants.

DISCUSSION

One of the major challenges in agriculture worldwide is to control the great yield loss caused by pests and fungal pathogens. Realization of this objective, however, in an environmental-friendly way necessitates integrated efforts from plant breeders, pathologists and genetic engineers. A major application of gene transfer technology is the introduction of agronomically useful traits into crop plants. Genes of agronomic importance such as those that confer resistance to disease and insects have been isolated from plants and other microorganisms. Establishment of a high-frequency regeneration system is an essential prerequisite for generation of transgenic plants. In this investigation, an efficient highly improved protocol for plant regeneration from shoot-tip-derived embryogenic calli of pearl millet, var. CO9, has been established. Starting from a single shoot-tip explant, >500 plantlets could be regenerated within 55 days which offers ample scope for adopting this technique for successful genetic transformation of pearl millet.

Recently established efficient transformation systems for improvement of cereals have made it possible to test the general usefulness of these genes in protecting food crops. Pearl millet is one of the important coarse cereal crops for which efficient transformation system has not been available (Vasil, 2008). We had previously studied various factors influencing the *Agrobacterium*- mediated transformation with improved transformation efficiency of two cultivars of pearl millet (unpublished data). Though, cultures of pearl millet are known to be recalcitrant to *in vitro* manipulation, we have been able to achieve high transformation efficiency with highly optimized conditions necessary for successful transformation of pearl millet. Transgenic pearl millet lines derived from CO9 genotypes were generated in this study. The significant number of GUS positive independent transgenic lines showed improved transformation frequencies of 4.5-6.5. This indicated that the *Agrobacterium*-mediated transformation enhanced transformation efficiency compared to other direct methods (Arockiasamy and Ignacimuthu, 2007; Gasparis *et al*., 2008; Ceasar and Ignacimuthu, 2011).

Fungal diseases constitute a major challenge to millions of pearl millet farmers throughout the tropical

regions where pearl millet is grown. Rust is one of the major biotic constraints in pearl millet production (Rachie and Majmudar, 1980; Girgi *et al*., 2006). Only recently transgenic pearl millet plants with improved agronomic traits have been produced by introducing useful genes such as pin and afp for downey mildew and rust resistance, respectively (Latha *et al*., 2006; Girgi *et al*., 2006). The transformation rates achieved using these methods are low compared to other cereals such as rice, maize and wheat (Christou *et al*., 1992; Becker *et al*., 1994; Brettschneider *et al*., 1997).

This study also proved the efficiency and effectiveness of the super virulent *Agrobacterium* tumefaciens LBA 4404 (pSB1/pKAN-Rchit 1.1) in transforming pearl millet. The plasmid pSB1 contained extra copies of vir genes, thus broadening the choice of *Agrobacterium* for monocot transformation. It has been suggested that the presence of additional vir gene sequence may be important to transform pearl millet cultivars as well as increase the transformation efficiency (Gelvin, 2000). In this study we report for the first time a significant enhancement of fungal resistance in pearl millet. Similar superbinary vectors have been used in other crop plants. Previous reports have also shown elevated chitinase activity in transgenic canola (Brogue *et al*., 1991), strawberry (Asao *et al*., 1997), rice (Nishizawa *et al*., 1999), tobacco (Broglie *et al*., 1989; Carstens *et al*., 2003), cotton (Emani *et al*., 2003) and Italian ryegrass (Takahashi *et al*., 2005) enhancing resistance to fungal diseases, although the level of chitinase activity does not always correlate with the degree of disease resistance (Nishizawa *et al*., 1999; Emani *et al*., 2003).

The transgenic plants exhibited normal growth in terms of phenotype and yield of seeds. PCR and Southern hybritization analysis proved the integration of the transgenes into the pearl millet genome. The copy number of transgene varied from 1-2. Multiple gene copies might cause unstable inheritance and transgene silencing; therefore transgenic plants with single copy insertion are more important (Ye *et al*., 2009; Ignacimuthu and Raveendar, 2010). PCR analysis proved the integration of the transgene and none of the amplification of the sequences beyond the T-DNA border was seen when total DNA was subjected to PCR analysis with nptII and chitinase primers. Southern blot analysis with Hind III digested DNA suggested that all the six transgenic lines showed expected 3.1 kb size, indicating the integration of the rice chitinase gene into the genome of pearl millet and proved that they were derived from independent transformants. Segregation of the chitinase gene in the next generation was examined by kanamycin resistance and GUS assay experiments. Segregation analysis of these transgenics (independent T_0 lines) demonstrated that the transgenes were stably inherited to T_1 progeny.

Fungal bioassays on T_1 progenies of three primary transformants, untransformed control (CO9) and susceptible check were done using a highly virulent rust fungal pathotype. Initially the disease symptoms appeared as chlorosis at the base of leaf lamina on the second leaf of susceptible T_1 seedlings, untransformed control and susceptible check. The disease symptoms developed in all the subsequent leaves showed the progression of severe damage caused with the pathogen giving a rusty appearance. All susceptible seedlings were invariably stunted and eventually died. Conversely the resistant T_1 seedlings which grew rapidly, did not show any of the disease symptoms; they were healthy and attained maturity with normal seed fertility. The results of bioassay study revealed a significant reduction of the spore germination sprayed on transgenic pearl millets than the untransformed control plants. The results of fungal bioassays on T_1 progenies of three transformants amply testified that the expression of chit11gene in transgenic pearl millet imparted high-level of resistance against *P. penniseti*. The transgene used in other crops viz., rice (Sridevi *et al*., 2005) and italian ryegrass (Takahashi *et al*., 2005) showed enhanced resistance to fungal pathogen.

CONCLUSION

In conclusion, transgenic pearl millet plants were regenerated utilizing optimized *Agrobacterium* cocultivation and selection conditions. GUS staining and chit11 gene southern blot analyses confirmed the successful introduction, integration, inheritance and Mendelian R1 segregation of transgenes in pearl millet. The introduced chit11 gene was found to be stably integrated into the genome of pearl millet. The transgenic pearl millet plants showed improved fungal resistance to rust pathogen, *P. penniseti*. Many protocols were developed and further refined during the earlier period for the *Agrobacterium*-mediated transformation of monocot cereals. Many of these transgenic cereals have previously reached the field for large-scale cultivation. However, a genetic improvement program for millets has been initiated only in recent years and has received less attention despite their nutritional importance. No report is available till date for *Agrobacterium*-mediated transformation of pearl millet (Ceasar and Ignacimuthu, 2009). This study proved the amenability of pearl millet to *Agrobacterium*-mediated gene transfer and development

of transgenic plants. The system developed here may be utilized in future for inserting many agronomically important genes into pearl millet.

ACKNOWLEDGMENTS

We thank the University Grants Commission, (New Delhi) for financial support. We gratefully acknowledge Dr. S. Muthukrishnan, Kansas State University, USA for providing the plasmid harbouring rice chitinase gene and Dr. R. Terada, National Institute of Basic Biology, Japan for providing the binary vector for cloning. We thank Tamil Nadu Agricultural University, Madurai, for assistance in fungal bioassay. We thank Tamil Nadu Agriculture University, Coimbatore and ICRISAT, AP, for proving seed materials. We are greatful to Dr. K. Veluthambi, School of Biotechnology, Madurai Kamaraj University, Madurai for technical training. We thank Dr. M. Gabriel Paulraj, Scientist, Entomology Research Institute, Loyola College for guidance. We thank Mr. M. Ramakrishnan, ERI, Loyola, College, for his Scientific support.

REFERENCES

Anonymous, 1996. The World Sorghum and Millet Economies: Facts, Trends and Outlook. Food and Agriculture Organization, Rome, Italy, Pages: 68.

Arockiasamy, S. and S. Ignacimuthu, 2007. Regeneration of transgenic plants from two indica rice (*Oryza sativa* L.) cultivars using shoot apex explants. Plant Cell Rep., 26: 1745-1753.

Asao, H., Y. Nishizawa, S. Aria, T. Sato and M. Hirai *et al*., 1997. Enhanced resistance against a fungal pathogen Sphaerotheca humuli in transgenic strawberry expressing a rice chitinase gene. Plant Biotechnol., 14: 145-149.

Becker, D., R. Brettschneider and H. Lorz, 1994. Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. Plant J., 5: 299-307.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Brettschneider, R., D. Becker and H. Lorz, 1997. Efficient transformation of scutellar tissue of immature maize embryos. Theor. Appl. Genet., 94: 737-748.

Broglie, K.E., P. Biddle, R. Cressman and Broglie, 1989. Functional analysis of DNA sequences responsible for ethylene regulation of a bean chitinase gene in transgenic tobacco. Plant Cell, 1: 599-607.

Brogue, K., I. Chet, M. Holliday, R. Cressman and P. Biddle *et al*., 1991. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science, 254: 1194-1197.

Carstens, M., M.A. Vivier and I.S. Pretorius, 2003. The *Saccharomyces cerevisiae* chitinase, encoded by the CTS1-2 gene, confers antifungal activity against *Botrytis cinerea* to transgenic tobacco. Transgenic Res., 12: 497-508.

Ceasar, S.A. and S. Ignacimuthu, 2009. Genetic engineering of millets: Current status and future prospects. Biotechnol. Lett., 31: 779-788.

Ceasar, S.A. and S. Ignacimuthu, 2011. *Agrobacterium*-mediated transformation of finger millet (*Eleusine coracana* (L.) Gaertn.) using shoot apex explants. Plant Cell Rep., 30: 1759-1770.

Ceasar, S.A. and S. Ignacimuthu, 2012. Genetic engineering of crop plants for fungal resistance: Role of antifungal genes. Biotechnol. Lett., 34: 995-1002.

Chen, L., S. Zhang, R.N. Beachy and C.M. Fauquet, 1998. A protocol for consistent, large -scale production of fertile transgenic rice plants. Plant Cell Rep., 18: 25-31.

Chilton, M.D., T.C. Currier, S.K. Farrand, A.J. Bendich, M.P. Gordon and E.W. Nester, 1974. *Agrobacterium tumefaciens* DNA and P58 bacteriophage DNA not detected in crown gall tumours. Proc. Natl. Acad. Sci. USA, 71: 3672-3676.

Christou, P., T.L. Ford and M. Kofron, 1992. Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important Indica and Japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Biol. Technol., 9: 957-962.

Devi, P. and M. Sticklen, 2002. Culturing shoot-tip clumps of pearl millet (*Pennisetum glaucum* (L.) R. Br.) and optimal microprojectile bombardment parameters for transient expression. Euphytica, 125: 45-50.

Devi, P., H. Zhong and M.B. Sticklen, 2000. *In vitro* morphogenesis of pearl millet (*Pennisetum glaucum* (L.) R.Br.]: efficient production of multiple shoots and inflorescences from shoot apices. Plant Cell Rep., 19: 546-550.

Dixon, R.A., 2001. Natural products and plant disease resistance. Nature, 411: 843-847.

Emani, C., J.M. Garcia, E. Lopata-Finch, M.J. Pozo and P. Uribe *et al*., 2003. Enhanced fungal resistance in transgenic cotton expressing an endochitinase gene from *Trichoderma virens*. Plant Biotechol. J., 1: 321-336.

Fisher, R.A., 1935. The Design of Experiments. Oliver and Boyd, Edinburgh.

FAO., 2004. Food and agricultural indicators. FAO Statistics Division. http://www.fao.org/es/ess/compendium_2004/concepts.asp

Gasparis, S., S. Bregier, W. Orczyk and A. Nadolska-Orczyk, 2008. *Agrobacterium*-mediated transformation of oat (*Avena sativa* L.) cultivars via., immature embryo and leaf explants. Plant Cell Rep., 27: 1721-1729.

Gelvin, S.B., 2000. *Agrobacterium* and plant genes involved in T-DNA transfer and integration. Annu. Rev. Plant Physiol. Plant Mol. Biol., 51: 223-256.

Girgi, M., M.M. O'Kennedy, A. Morgenstern, G. Smith, H. Lorz and K.H. Oldach, 2002. Transgenic and herbicide resistant pearl millet (*Pennisetum glaucum* L.) R.Br. via., microprojectile bombardment of scutellar tissue. Mol. Breed., 10: 243-252.

Girgi, M., W.A. Breese, H. Lorz and K.H. Oldach, 2006. Rust and downy mildew resistance in pearl millet (*Pennisetum glaucum*) mediated by heterologous expression of the afp gene from *Aspergillus giganteus*. Transgenic Res., 15: 313-324.

Goldman, J.J., W.W. Hanna, G. Fleming and P. Ozias-Akins, 2003. Fertile transgenic pearl millet ((*Pennisetum glacum* (L.) R. Br.) plants recovered through microprojectile bombardment and phosphophenothricin selection of apical meristem, inflorescence and immature embryo derived embryogenic tissues. Plant Cell Rep., 21: 999-1009.

Grover, A. and D. Pental, 2003. Breeding objectives and requirements for producing transgenics for major field crops of India. Curr. Sci., 84: 310-320.

Gueye, M.T. and A. Delobel, 1999. Relative susceptibility of stored pearl millet products and fonio to insect infestation. J. Stored Prod. Res., 35: 277-283.

Hain, R., H.J. Reif, E. Krause, R. Langebartels and H. Kindl *et al*., 1993. Disease resistance results from foreign phytoalexin expression in a novel plant. Nature, 361: 153-156.

Hoagland, D.R. and D.I. Arnon, 1950. The water culture methods for growing plants without soil. University of California, Agricultural Experiment Station, Berkeley, Circular No. 347. http://www.ars.usda.gov/SP2UserFiles/Place/36071000/GPRG/WaterCultureMethodForGrowingPlantsWithoutSoil.pdf

Ignacimuthu, S. and S. Raveendar, 2010. *Agrobacterium* mediated transformation of indica rice (*Oryza sativa* L.) for insect resistance. Euphytica, 179: 277-286.

Jefferson, R.A., 1987. Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep., 5: 387-405.

Kishimoto, K., Y. Nishizawa, Y. Tabei, T. Hibi, M. Nakajima and K. Akutsu, 2002. Detailed analysis of rice chitinase gene expression in transgenic cucumber plants showing different levels of disease resistance to gray mold (*Botrytis cinerea*). Plant Sci., 162: 655-662.

Lambe, P., M. Dinant and R.F. Matagne, 1995. Differential long-term expression and methylation of the hygromycin phosphotransferase (hph) and β-glucuronidase (GUS) genes in transgenic pearl millet (*Pennisetum glaucum*) callus. Plant Sci., 108: 51-62.

Lambe, P., M. Dinant and R. Deltour, 2000. Transgenic Pearl Millet (*Pennisetum glaucum*). In: Biotechnology in Agriculture and Forestry, Bajaj, Y.P.S. (Ed.). Springer, New York, USA., pp: 84-108.

Latha, A.M., K.V. Rao, T.P. Reddy and V.D. Reddy, 2006. Development of transgenic pearl millet (*Pennisetum glaucum* (L.) R. Br.) plants resistant to downy mildew. Plant Cell Rep., 25: 927-935.

Mauch, F., B. Mauch-Mani and T. Boller, 1988. Antifungal hydrolases in pea tissue: II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. Plant Physiol., 88: 936-942.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant., 15: 473-497.

Nishizawa, Y., Z. Nishio, K. Nakazono, M. Soma, E. Nakajima, M. Ugaki and T. Hibi, 1999. Enhanced resistance to blast (*Magnaporthe grisea*) in transgenic Japonica rice by constitutive expression of rice chitinase. Theor. Applied Genet., 99: 383-390.

O'Kennedy, M.M., H.C. Stark and N. Dube, 2011. Biolistic-mediated transformation protocols for maize and pearl millet using pre-cultured immature zygotic embryos and embryogenic tissue. Methods Mol. Biol., 710: 343-354.

O'Kennedy, M.M., J.T. Burger and F.C. Botha, 2004a. Improved regeneration efficiency of a pearl millet (*Pennisetum glaucum*) breeding line. S. Afr. J. Bot., 70: 502-508.

O'Kennedy, M.M., J.T. Burger and F.C. Botha, 2004b. Pearl millet transformation system using the positive selectable marker gene phosphomannose isomerase. Plant Cell Rep., 22: 684-690.

Rachie, K.O. and J.V. Majmudar, 1980. Pearl Millet. Pennsylvania State University Press, Pennsylvania, USA., Pages: 307.

Rai, K.N., K. Anand Kumar, D.J. Andrews, S.C. Gupta and B. Ouendeba, 1997. Breeding pearl millet for grain yield and stability. Proceedings of the International Conference on Genetic Improvement of Sorghum and Pearl Millet, September 22-27, 1996, Holiday Inn Plaza, Lubbock, Texas, pp: 481-489.

Roger, S.O. and A.J. Bendich, 1994. Extraction of Total Cellular DNA from Plants, Algae and Fungi. In: Plant Molecular Biology Manual Belgium, Gelvin, S.B. and R.A. Schilperoot (Eds.). Kluwar Academic Publishers, London, pp: 1-8.

Sharma, K.K. and R. Ortiz, 2000. Program for the application of genetic transformation for crop improvement in the semi-arid tropics. *In vitro* Cell Dev. Biol. Plant., 36: 83-92.

Singh, S.D., S.B. King and J. Werder, 1993. Downy mildew disease of pearl millet. Information Bulletin No. 37. Technical Report. International Crops Research Institute for the Semi-Arid Tropics, Patancheru andhra Pradesh, India, pp: 1-30.

Southern, E.M., 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol., 98: 503-517.

Sridevi, G., M. Dhandapani and K. Veluthambi, 2005. *Agrobacterium*-mediated transformation of White Ponni, a non-basmati variety of indica rice (*Oryza sativa* L.). Curr. Sci., 88: 128-132.

Takahashi, W., M. Fujimori, Y. Miura, T. Komatsu, Y. Nishizawa, T. Hibi and T. Takamizo, 2005. Increased resistance to crown rust disease in transgenic Italian ryegrass (*Lolium multiflorum* Lam.) expressing the rice chitinase gene. Plant Cell Rep., 23: 811-818.

Taylor, M.G. and I.K. Vasil, 1991. Histology of and physical factors affecting, transient GUS expression in pearl millet (*Pennisetum glaucum* (L.) R. Br.) embryos following microprojectile bombardment. Plant Cell Rep., 10: 120-125.

Taylor, M.G., V. Vasil and I.K. Vasil, 1993. Enhanced GUS gene expression in cereal/grass cell suspensions and immature embryos using the maize uhiquitin-based plasmid pAHC25. Plant Cell Rep., 12: 491-495.

Thakur, R.P. and K. Mathur, 2002. Downy mildews of India. Crop Protec., 21: 333-345.

Toriyama, K. and K. Hinata, 1985. Cell suspension and protoplast culture in rice. Plant Sci., 41: 179-183.

Van Loon, L.G. and E.A. van Strien, 1999. The families of pathogenesis-related proteins, their activities and comparative analysis of PR-1 type proteins. Physiol. Mol. Plant Pathol., 55: 85-97.

Vasil, I.K., 2008. A short history of plant biotechnology. Phytochem. Rev., 7: 387-394.

Wilson, J.P., 2000. Pearl Millet Disease: A Compilation of Information on the Known Pathogens of Pearl Millet, *Pennisetum glacum* (L.) R. Br. U.S. Department of Agriculture, Economic Research Service, Washington, DC., USA., Pages: 50.

Ye, R., H. Huang, Z. Yang, T. Chen and L. Liu *et al*., 2009. Development of insect-resistant transgenic rice with Cry1C*-free endosperm. Pest Manage. Sci., 65: 1015-1020.

Zhu, Q., E.A. Maher, S. Masoud, R.A. Dixon and C.J. Lamb 1994. Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic Tobacco. Nat. Biotechnol., 12: 807-812.