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Research Article

An efficient high throughput plant regeneration and transformation protocol for production of transgenics tolerant to salt in finger millet

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Abstract : Finger millet [*Eleusine coracana* (L.) Gaertn.] is the primary food source for millions of people in tropical dry land regions of the world. Development of efficient and genotype-independent tissue regeneration system is an essential prerequisite for successful production of transgenic plants. In this direction we attempted *in vitro* plant regeneration and transformation study in finger millet crop using *PDH45* as a candidate gene to develop transgenics for salt tolerance using *Agrobacterium* mediated gene transfer method (*in vitro* method). Here we used actively dividing embryogenic seed calli as explantt. The seed calli was co cultivated with *Agrobacterium* carrying binary vector pCAMBIA contains PDH45 gene, *nptII, hptII* , and *GUS* reporter gene driven by CaMV 35S promoter. The co cultivated callus was regenerated in half strength MS media with 0.5 mg.L^{-1}BA, 3.0 mg.L^{-1} 2, 4-D, and hygromycin antibiotic supplemented with acetosyringone (100 mg.ml^{-1}), a potent inducer of virulence genes. Successful transformation at callus stage was initially confirmed by *GUS* histochemical assay. By PCR amplification genomic DNA of putative transformed calli showed positive for *hptII* primers. The results by RT-PCR showed that the level of transcripts overexpression in transformed calli was relatively higher than nontransformed control calli. The regenerated transgenic plants were confirmed by PCR amplifying the genomic DNA.

Key Words : Agrobacterium, Callus, Finger millet, *PDH45*, Regeneration

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INTRODUCTION

Among eight minor millets, finger millet [*Eleusine coracana* (L.) Gaertn.], also known as African millet, has outstanding attributes as a subsistence food crop. It is grown globally in more than 4 million ha. and is the primary food source for millions of people in tropical dryland regions. Finger millet constitutes about 81 per cent of the minor millets produced in India. Finger millets also have nutritional qualities superior than that of rice and is at par with that of wheat (Sastri, 1989).

The first experiments to culture plant cells under *in vitro* conditions were conducted more than one hundred years ago (Haberlandt, 1902). It took decades until their detection, isolation and subsequently the observation made by Skoog and Miller (1957) on the auxin/cytokinin ratio controlling root and/or shoot formation from tobacco (*Nicotiana tabacum*) pith tissue cultures *in vitro*, being a milestone for the development of plant tissue and cell culture. However, more than twenty years after that breakthrough and promising results with dicots, success with monocots, especially with the cereals was rare (King *et al.*, 1978). Plant regeneration has been reported to

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occur routinely in almost all cultivated cereal species (Vasil, 1987; Bhaskaram and Smith, 1990; Kothari and Chandra, 1995). In finger millet, callus formation and plantlet regeneration was first reported by (Rangan, 1976), from mesocotyl explants. Genetic transformation is now widely used as a method of choice for transferring exotic genes into commercial crop cultivars for enhancing various agronomic attributes. So far, limited attempts have been made in finger millet to standardize protocols for genetic transformation (Gupta *et al.,* 2001).

Salinity is currently the major factor which reduces crop yields. World-wide about 33 per cent of the irrigated land is affected by salinity and more land is not being irrigated because of salinity (Marschner, 1993; Chaves *et al.*, 2009). Hence it's becoming most important abiotic stress factor in recent days and the total global area of salt affected soils including saline and sodic soils is 831 million hectares (Martinez-Beltran and Manzur, 2005). There is a reduction in plant height upto 40-52 per cent in finger millet varieties when subjected to salinity stress (150mM NaCl). There is considerable decrease in the contents of total sugar, reducing sugars, non-reducing sugars in finger millet under salt stress (Manikandan and Desingh, 2009). Zhu (2002) stated that at molecular level there are two main adaptive mechanisms for saline tolerance *viz.*, (i) homeostasis that includes ion homeostasis (ii) stress damage control by repair or detoxification. In recent days there is evidence that Helicases also can be involved in abiotic stress tolerance. Because salinity stress affects the cellular gene-expression machinery it is evident that molecules involved in nucleic acid processing, including helicases, are likely to be affected as well (Neeti-Sanan-Mishra *et al.*, 2005). These DNA Helicases unwind duplex DNA and hence involved in replication, repair, recombination, and transcription regulation machinery of the cell. Where as RNA helicases unfold the secondary structures in RNA (Owttrim, 2006). And these are involved in transcription, ribosome biogenesis, and translation initiation, RNA editing, and development (Tuteja *et al.*, 1996).

Mishra *et al.* (2005) found that overexpression of *PDH45* (Pea DNA helicase 45) in tobacco imparts salinity tolerance without affecting the yield. Hence, in this direction in finger millet we established efficient reproducible protocols for *in vitro* plant regeneration and genetic transformation using *PDH45* candidate gene to develop finger millet transgenics for salinity tolerance by *Agrobacterium* mediated gene transfer method (*in vitro* method).

EXPERIMENTAL METHODS

The binary vector pCAMBIA:PDH45 gene construct was obtained from Dr. Narendra Tuteja, ICGEB, New Delhi, India. This binary vector pCAMBIA:PDH45 harbors *PDH45* driven by CaMV 35S promoter. The binary vector pCAMBIA 1301 has *nptII* gene as bacterial selection marker and *hptII* gene as plant selectable marker. It was initially confirmed by restriction analysis. Binary vector pCAMBIA1301 was transformed into *E. coli* (DH5α) competent cells (Competent cells were prepared by KCM method). The plasmid was isolated from *E.coli* (Alkali lysis method), and mobilized into *Agrobacterium tumefaciens* (strain LBA4404) by electroporation (Bio-Rad Laboratories Ltd).

Electroporation procedure:

Frozen cells were thawed on ice and 40µl aliquot was transferred to a precooled 0.2cm electroporation cuvette (Bio-Rad Laboratories Ltd.). One µl of plasmid DNA (2-10ng) was mixed with the cell suspension on ice and an electric pulse applied immediately using a gene pulser^{Tm} with pulse controller unit (Bio-Rad). The cells were immediately transferred to 1 ml YMB or TY and shaken at 29^{0}C for 3hrs. Aliquots of 10µl or 100µl were plated on LB media containing antibiotics and incubated for 3d at 29^{0}C.

Mobilization of *E. coli* plasmid in to *Agrobacterium* was confirmed by PCR analysis using *PDH45* gene specific primers. The primers which were designed using DNA STAR programme based on *PDH45* m-RNA sequence.

Primer sequence for PDH45:

PDH45 forward Primer: 5' TCCTGGGCGAGTCTGTGA3'

PDH45 reverse primer: 5' CTCCCATAATTGCATCTCT-TTCTT3'

After successful mobilization into *Agrobacterium*, *PDH45* gene was transformed into finger millet (GPU-28 variety) by using (*in vitro) Agrobacterium* mediated gene transfer method. For this we used actively dividing embryogenic finger millet seed callus as explant for transformation.

Callus induction:

Seeds of *Eleusine coracana* were used as source material for callus initiation. Seeds were surface sterilised in 70 per cent (v:v) ethanol for 3 min followed by 0.1 per cent (w:v) $HgCl_2$ solution for 5 min, rinsed several times in sterile distilled water and cultured aseptically. Callus induction media containing basal MS medium containing 3 per cent sucrose with growth hormones 0.5 mg.L^{-1} BA, 3.0 mg.L^{-1} 2,4-D was used. The medium was solidified with 0.8 per cent agar (Bacteriological grade), pH adjusted to 5.8 and then autoclaved at 1.2–1.3 kg.cm^{-2} pressure and 121°C temperature for 15 min.

Seeds were inoculated in callus induction media, incubated in dark with 26±1°C temperature condition for 3-4 weeks in growth chamber. Later the induced callus was transferred in to the callus growth media containing 2mg.L^{-1} 2, 4-D for callus grown for 2 weeks. After 2 weeks of incubation the compact, green, nodulated sectors of callus were separated from non embryogenic watery callus and then subcultured on MS medium with lower level of 2, 4-D (0.2 mg.L^{-1}). The embryogenic callus was subcultured every 3–4 weeks on fresh medium for

maintaining the same in embryogenic state. The embryogenic callus developed after 5–6 passages was used for plant gene transformation.

Infection and co- cultivation *(in vitro* method):

The starter culture of *Agrobacterium tumefaciens* strain LBA-4404 plasmid carrying binary vector pCAMBIA:PDH45 construct was grown in AB minimal media supplemented with kanamycin (50 µg.mL^{-1}). Then calli explants were soaked in bacterial suspension for 3-4 minutes for infection. After infection calli were blotted on the sterile tissue paper and then transferred to co culture medium for 2 days. Co culture medium was prepared by using MS media with 3.0mg.L^{-1} 2, 4-D and 0.5mg.L^{-1} BA, supplemented with acetosyringone. During co cultivation *Agrobacterium* was found to grow on and around the callus explants. After co cultivation period, calli were washed with cefotaxime (200µg.ml^{-1}) to remove excess bacteria and blotted on sterile tissue paper before transferring them to selection media. (This selection media was prepared using MS media supplemented with 1.0 mgL^{-1} BA, 30 µg.mL^{-1} hygromycin and 40µg.mL^{-1} of cefotaxime). Cefotaxime was used to kill the excess *Agrobacterium* after infection, and hygromycin was used to select the transformants.

After 2 days the *Agrobacterium* colonies adhered onto the calli was washed with cefotoxime (50µg.ml^{-1}) and kept in dark on regeneration media containing 50ìg.ml^{-1} cefotoxime and 30ìg.ml^{-1} hygromycin for three weeks. Later these regenerated calli was incubated under light. The calli surviving on selection media were transferred into the regeneration media contained half strength MS basal with 0.5 mg.L^{-1} BA, 3.0mg.L^{-1} 2, 4-D, 30 ìg.mL^{-1} hygromycin and 40µg.mL^{-1} of cefotaxime for production of root and shoots. The uninfected (non-cocultivated) control calli was grown in finger millet regeneration media to maintain a positive control and to finger millet selection media to maintain a negative control.

Confirmation of putative transformants by molecular analysis:

Initially putative transformed calli were confirmed by Gus-histochemical analysis. Proliferated callus was obtained from the putative transformed calli were immersed in GUS staining solution and incubated overnight at 37^{0}C. After removing the pigments by 70-100 per cent ethanol, GUS expression cells were detected microscopically by a distinct blue coloration due to enzymatic cleavage of 5-bromo-4-chloro-3-indolyl glucuronide. The DNA was extracted (C-TAB method) from putative transformed calli and the transformation was reconfirmed by PCR analysis using *nptII* antibiotic primers.

To compare the level of transcript expressed between transformed and non-transformed calli lines, equal amount of total RNA was used from transformed and control calli to perform a reverse transcription PCR. The cDNA was made using oligodT primers. PDH45 and actin transcripts were amplified by using equal quantity of cDNA. The RT-PCR products were analysed on 0.8 per cent agarose gel electrophoresis

Genomic DNA from regenerated plant leaves was isolated from putative transformants and untransformed finger millet (negative control). And the event of successful transformation in finger millet was examined by testing for the presence of the *hptII* and PDH45 coding gene sequence in the by PCR analysis.

EXPERIMENTAL RESULTS AND ANALYSIS

pCAMBIA:PDH45 construct (Fig.1) was obtained in the form of plasmid and it was initially confirmed by restriction analysis. The plasmid obtained was first transformed into competent *E. coli* (DH5α) cells and multiplied and confirmed. Then the binary vector plasmid was mobilized into *Agrobacterium* strain LBA4404 by electroporation.

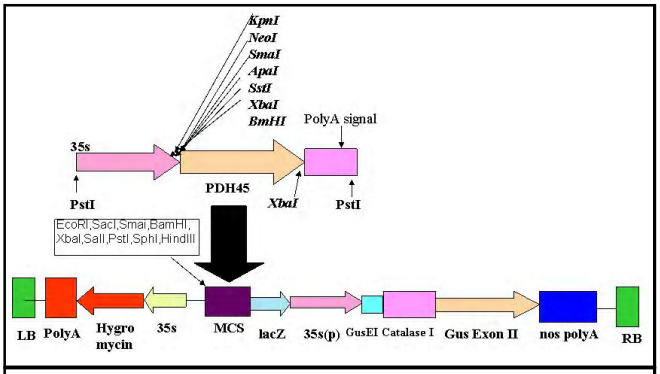

Fig. 1: Binary vector pCAMBIA:PDH45 gene construct

The per cent survival of transformed calli grown on regeneration media containing hygromycin plant selection media was recorded for each batch of co cultivation. And we found that the transformation efficiency was around one per cent (Table 1). Since the finger millet is monocot there is less wound response and absence of associated activation of virulence genes makes regeration efficiency is comparatively

Table 1 : Per cent survival of agro co-cultivated calli on regeneration media containing hptII (Seven batches was co-cultivated with *Agrobacterium*)

Batch no.	Number of calli co cultivated with Agrobacterium strain	Number of calli survived in hygromycin selection media	Per cent survival
1	106	01	0.94
2	89	02	2.24
3	84	01	1.19
4	101	02	1.98
5	126	02	1.58
6	136	01	0.73
7	149	01	0.67

less than dicot plants.

The PDH-45 gene was transformed into finger millet seed calli by *Agrobacterium* mediated gene transfer method (*i.e. in vitro* method). Transformed callus was grown in selection media and kept for regeration. Here the nontransformed embryonic calli were unable to regenerate in *nptII* antibiotic media and was killed. Whereas *nptII* gene containing calli was regerated since it gives resistance to antibiotic (Fig. 2A). And this transformation initially confirmed by Gus-histochemical analysis. The transformed calli showed distinct blue coloration due to enzymatic cleavage of 5-bromo-4-chloro-3-indolyl glucuronide substrate (Fig. 2B).

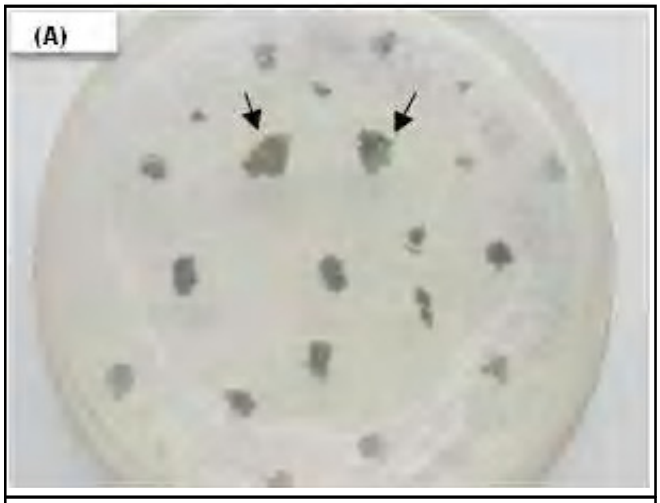

Fig. 2A : Selection and regeneration of putative transformed calli in selection media. Arrow mark indicates the survived calli in selection media

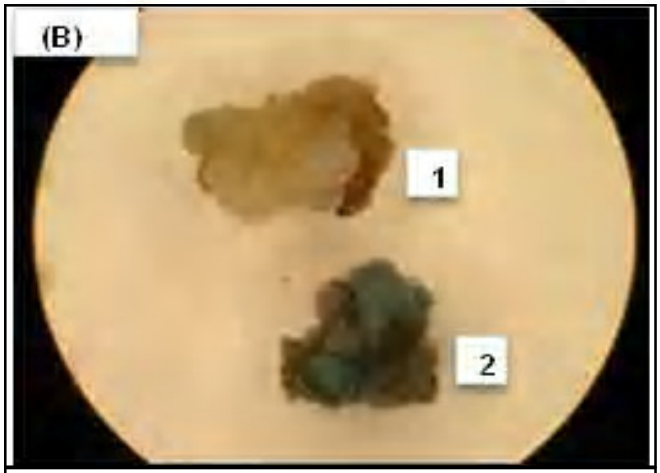

Fig. 2B: Confirmation of the putative transformants at callus stage by Gus assay using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide as substrate. 1=Control (non-transformed) callus, 2 =Putative transformed callus

Molecular analysis of the putative *PDH45* transformants:

The genomic DNA was extracted from both untransformed control callus and putative *PDH45* transformant callus by C-TAB method. Using the genomic DNA as template, PCR was performed for *hptII* (selectable marker). The DNA of untransformed finger millet callus line did not give any amplification for *hptII* primers (Fig.3, lane 1); suggesting that the untransformed control plants did not carry any *hptII* gene encoding for hygromycin resistance. And the putative transformants showed the amplification of 500bp *hptII* fragment (Fig.3, lane 3).

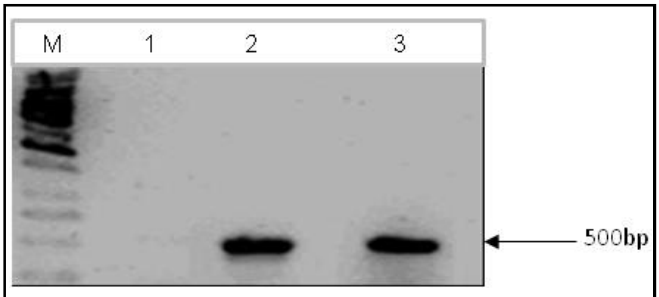

Fig 3: Confirmation of putative transformed callus over-expressing PDH45 by PCR analysis of callus genomic DNA using hptII primers.
Lane M: Gene Ruler 1kb ladder
Lane 1: Genomic DNA of untransformed control callus
Lane 2: Plasmid DNA of pCABIA1301-*PDH45* construct
Lane 3: Genomic DNA of putative transformed calli over expressing *PDH45* gene

Semi-quantative RT-PCR was used to analyze the expression of transgenic callus at the RNA level. The RT-PCR result indicated that expression of *PDH45* transcripts was more in putative transformed line when compared to control (Fig. 4). But even in non-transformed calli there was negligible *PDH45* transcript amplification was observed this is because *PDH45*

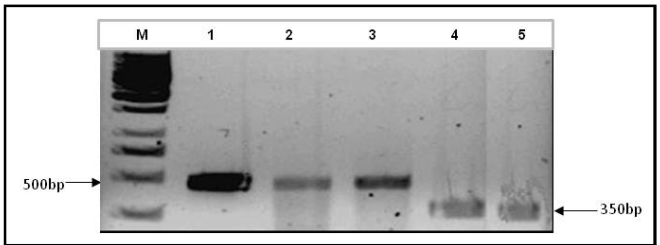

Fig 4 : Amplification of PDH45 from cDNA of putative transformed Finger millet callus
M: Gene Ruler 1kb ladder
Lane 1: Plasmid DNA of pCABIA1301-*PDH45* construct
Lane 2: Amplification of *PDH45* from cDNA of non-transformed control calli
Lane 3: Amplification of *PDH45* from cDNA of PDH 45 transformed finger millet calli
Lane 4 and 5: Actin amplification of Lane2 and 3

is a functional gene so it endogenously expresses even in control plants also. This suggest the over expression of *PDH45* gene in transformed line.

The genomic DNA was obtained from regenerated plant leaves putative *PDH45* transformed plants and non-transformed control finger millet plant by C-TAB method. And the PCR result which shown that non-transformed finger millet line did not give any amplification for *hptII* primers (Fig. 5, lane 1); Suggesting that the control plants did not carry any *hptII* gene encoding for hygromycin resistance. The genomic DNA of the independent putative *PDH45* transformed plant showed the amplification for *hptII* fragment primers (Fig. 5, Lane 2).

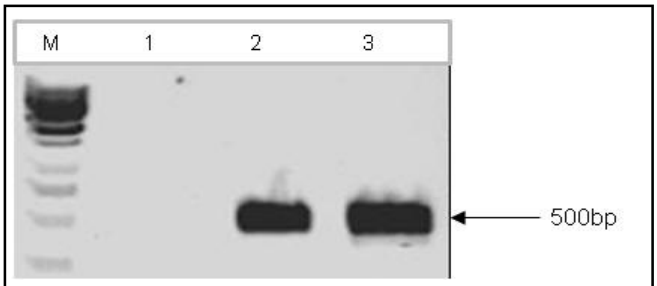

Fig. 5: Confirmation of successful in vitro transformed Finger millet transgenic lines over-expressing PDH 45 gene by PCR analysis of genomic DNA using hptII primers
Lane M: Gene Ruler 1kb ladder
Lane 1: Genomic DNA of control
Lane 2: Genomic DNA of putative transgenic plant over expressing *PDH45* gene
Lane 3: Plasmid DNA of pCAMBIA1301-*PDH45* construct

From these results it clearly indicates that, the *PDH45* gene has been successfully introduced into finger millet by *Agrobacterium* mediated gene transformation method.

Abbreviations used:

2,4-D: 2,4-Dichlorophenoxyacetic acid, BA: 6-Benzyladenine, *GUS*: b-Glucuronidase, *nptII*: Neomycin phosphotransferase II, PDH45: Pea DNA Helicase 45, *hptII*: Hygromycin phosphotransferase

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