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Transformation of mung bean plants for abiotic stress tolerance by introducing *codA* gene, for an osmoprotectant glycine betaine

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ABSTRACT

Mung bean (*Vigna radiata* L. Wilczek) is a major grain legume extensively cultivated in equatorial and semi-tropical regions of the Indian subcontinent as well as in South East Asian countries. Protein and carbohydrate of mung bean are easily digestible and create less flatulence than proteins derived from other legumes. Mung bean is very sensitive to salty, and dessicated soil and variations of temperature (very low or very high), during the flowering and seed/pod development stages resulting in heavy losses to productivity. The development of plants by the addition and over expression of preferred abiotic stress tolerant genes through genetic transformation suggested to be a usable choice for obtaining abiotic stress tolerant plants. Stable transformation and expression of transgene (*codA* gene) was achieved in mung bean through *Agrobacterium tumefaciens* mediated system using cotyledonary node explants, under the optimized situations. The genomic tests of putative transgenic plants were done through polymerase chain reaction, dot-blot, enzyme-linked immunosorbent assay and Western blotting. The primary transformants were checked for salt tolerance by the leaf disc test.

KEY WORDS: Abiotic stress, choline oxidase, glycine betaine

INTRODUCTION

Genetic transformation is a beneficial way for introducing genes from far-distant genomes, or from prokaryotes to eukaryotes and from the sources other than plants. the recent progress of transformation approach for producing abiotic stress resistance in plants has been reviewed by Sharma and Lavanya (2002), Wang *et al*. (2003), Flowers (2004), Vinocur and Altman, (2005), Chinnusamy *et al*. (2006), Yamaguchi and Blumwald (2005), Blumwald and Grover (2006), Bhatnagar-Mathur (2008). Various transgenic strategies used so far includes the transformation of the plants through genes that conceal for an important enzyme(s)/protein(s) involved in ion/proton transport, biosynthesis of certain osmoprotectants (Garg, *et al.,* 2002), scavengers of reactive oxygen species, proteins released during stress conditions like late-embryogenesis abundant proteins, signaling proteins in model plants such as arabidopsis, (Sakamoto, *et al.*, 2000) Rice, (Sakamoto, *et al.*, 1998, 2000; Mohanty *et al*. 2002; Kathuria *et al* 2009), Tobacco (Rathinasabapathi *et al*, 1994, 2000; Holmstrom, *et al* 2000; Huang, *et al* 2000) and Tomato (Park *et al* 2007; Goel *et al* 2011) rice, and tobacco.

The different approaches used by many plants and microorganisms to defend themselves with abiotic stress are to synthesis and accumulation of compounds known as osmoprotectants, generally, we called them compatible solutes. Glycine betaine (GB) is one of the powerful compatible solutes synthesized by plants during stress conditions (Bohnert *et al.*). It is nontoxic at higher concentration and raises the osmotic pressure in the cytoplasm (Le Rudulier *et al.*, 1984), stabilitate the framework and function of various proteins and enzymes (Sharma, Lavanya, *et al.,* 2002; Wang, 2003; Chandra *et al* .,2003; Flowers, 2004; Vinocur, Altman *et al*, 2005) Rontein *et al.*, 2002) and sustains the rectitude of membranes against the detrimental effects of freezing, heat and exaggerated salt conditions (Gorham, 1995; Sakamoto and Murata, 2000). In photosynthetic systems, GB effectively protects different constituents of the photosynthetic machinery such as, rubisco (ribulose-1, 5 biphosphate carboxylase/oxygenase) enzyme and prevents

photosystem II products from stress induced injuries (Papageorgious and Murata, 1995;). GB accumulates under stress conditions in many plants which are known as natural accumulator of GB and these plants belongs to the families, Chenopodiaceae and Poaceae. The amount of GB accumulation leads to increase tolerance of plants toward abiotic stress (Hayashi *et al*., 1997). External application of GB also enhances the vegetative and reproductive growth of several plants including natural accumulators and nonaccumulators under different stress conditions (Hayashi *et al*., 1997). The leaves taken from mature transgenic tomato plants confess the higher level of chlorophyll pigments and water and protein contents when compared with wild type plants under stress conditions (Zhang *et al*. 2011). Positive effects of GB in transgenic plants have also been reported by Chen and Murata (2011) and Cheng *et al*. (2013). Many important crops plants, e.g., tobacco, mung bean, rice, potato do not accumulate GB. Hence, genetic manipulation of these crop plants with genes responsible for GB biosynthesis could be an effective mode for developing stress tolerant plants (McCue and Hanson, 1990; Prasad *et al* 2000).

Among different pathways of GB synthesis, the most suitable target for metabolic engineering of GB is COD pathway that changes choline into GB in only one step because it involves the transfer of a single gene *codA*.

Engineering of GB biosynthesis in GB nonaccumulator leguminous crops including mung bean (*Vigna radiata*) are lacking in literature. Moreover, the appropriateness of this system toward abiotic stress resistance in leguminous plants is still unpracticed. Mung bean plants have been regenerated through, direct organogenesis by Chandra and Pal (1985), Mathew (1987), Gulati and Jaiwal (1992. p. 94), Tivarekar and Eapen (2001), Kumar *et al*. (2003), Vijayan *et al*. (2006), Mahalakshmi *et al*. (2006), and Mundhara and Rashid (2006), and indirect organogenesis by Patel *et al*. (1991), Mendoza *et al*. (1992), Amutha *et al*. (2003). However, the success of producing transgenics using these regeneration methods has been very low. Recently Sheng *et al* transformed codA gene in Brocolli (Sheng *et al* 2016). Only few reports are accessible on formation of transgenic plants in mung bean and all of them have employed *Agrobacterium* - mediated transformation (Sahoo and Jaiwal, 2008). Jaiwal *et al*. (2001) reported recovery of transgenic plants using the *nptII* gene which was used as a plant selectable marker with a frequency of 0.9% whereas Mahalakshmi *et al*. (2006) generated transgenics at a frequency of 2% using *hpt* gene.

Sonia *et al*., (2007) developed transgenic plants of mung bean by transforming an insecticidal α-amylase inhibitor gene for bruchid resistance and the *bar* gene for herbicide resistance with a frequency of 1.15%.

The current research was initiated with the aim to engineer the biosynthesis of GB into mung bean plants to intensify their resistance toward salinity and drought.

MATERIALS AND METHODS

Transformation

The *Agrobacterium tumefaciens* strain EHA101 carrying a binary vector pGAH/*codA* and cotyledonary node explants excised from 16 h old seedlings (presoaked in water) were used for the transformation studies. The explants after inoculated with bacterial strain were cultured on B_5 medium supplemented with 1.0 µm BAP, 75 mg/l kanamycin, 500 mgl^{-1} cefotaxime and 0.7% agar, for the regeneration of shoots. These explants were subcultured on a freshly prepared medium after every 2 weeks and maintained under the same conditions as for shoot regeneration. The green shoot (2-4 cm) recovered on kanamycin containing medium were transferred to B_5 basal medium having 2.5 µmIBA, 12.5 mgl^{-1} kanamycin and 350 mgl^{-1} cefotaxime for regeneration of roots. The shoots bearing fine roots were shifted to pots (for hardening) which were filled with soilrite (obtained from Kelperlite, Bengaluru, India). Pots were enclosed by polythene bags to retain high humid conditions for some days (Figure 1). The putative transgenic plants, which were well adapted in soil, were shifted to the green house and grown to maturity.

Molecular Analysis of Presumptive Transformants

Isolation of genomic DNA

Genomic DNA from putative transgenic and nontransgenic (control) plants was extricated by cetyltrimethylammonium bromide method given by Rogers and Bendich (1988).

Polymerase chain reaction (PCR) analysis

Putative transgenics were analyzed by PCR for detecting the presence of transgenes. The 0.54 kb region of *nptII* was intensified using the specific primers (Forward-5'-CCACCATGATATTCGGCAAC-3') and (Reverse-5'-GTGGAGGCTATTC GGCTA -3').

Every PCR reactions were performed in eppendrof tubes containing 25 µl of reaction solution having, 1 × reaction buffer, 200 µM dNTPs, 2 mM $MgCl_2$ and 100 ng of DNA, 1 µm of forward and reverse primers each and 1 unit of TaqDNA polymerase from MBI, Fermentas. The PCR

amplification of DNA was performed using a thermal cycler (Perkin-elmer), under the given conditions of temperature: 94°C for 5 min followed by 38 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min and final extension reaction at 72°C for 7 min.

Amplified DNA fragments were then analyzed by electrophoresis using agarose gel 1% and visualized through ethidium bromide staining (Sambrook *et al.*, 1989) under ultraviolet light. To confirm that reagents were not contaminated, the DNA from nontransformed plants was also examined in the experiment.

Dot-blot analysis

For dot-blot analysis, the DNA isolated from transformed and nontransformed plants was denatured by heating on a temperature 95°C for 5-7 min and cooled rapidly on ice. Dots of DNA were made with the help of a micropipette on nylon membrane. The membrane was allowed to dry and exposed to ultraviolet irradiation for 5 min. The membranes were then hybridized with dCT- [p^{32}] - labeled probes of *nptII* as well as *hpt* fragments at 65°C for 24 h. The membranes were washed at high rigorousness by deliberately reducing the salt from 5% standard saline citrate and 0.1% sodium dodecyl sulfate (SDS) to 0.1% SSS and 0.1% SDS. The membrane was disclosed to X-ray film for a sometime period before developing.

Protein extraction from plant material

Leaf tissue around 500 mg of fresh weight from 20 days old putative transgenic plants and from control plants were homogenized with the protein extraction buffer containing 40 mMTris-HCL (ph 7.2), 5 mM ethylenediaminetetraacetic acid and 10 mM 2-mercaptoethanolby using the mortar and pestle. The homogenate was centrifuged at 18,000 g for 15 min at 4°C. the supernatant was used for quantification of proteins by Bradford (1976) method and for other protein verifying techniques like western blot analysis.

Enzyme-linked immunosorbent assay (ELISA)

Total proteins were extracted from the leaves of transformed and nontransformed plants in the phosphate buffer having (pH 7.0) and poured into wells of 96 well ELISA plate (Bengaluru Genie) with other negative controls (only buffer, buffer + primary antibodies, only antigen, antigen + primary antibody, antigen + substrate). Active sites were blocked by treating with blocking solution (5% BSA in phosphate buffer saline) for 1 h at 37°C followed by washing with phosphate buffered saline (PBS) having 1% BSA. After that, plate was incubated with primary antibodies (1:1000 times) dilution for 1 h at around 25°C temperature and washed three times with PBST (1× PBS + 0.01 ×Tween 20). Then secondary antibodies (1:10000) were added for 1 h at normal room temperature and washed three times with PBST. At last, substrate 1-nitrophenyl (pNPP) was added and kept in dark for 30 min to visualize the color reaction.

SDS-polyacrylamide gel electrophoresis (PAGE) and Western analysis

20 µg of protein was partitioned on 10% of SDS and polyacrylamide-gel electrophoresis at 25 mA constant current in the presence of Tris-glycine-SDS buffer having 25 mM of Tris and 250 mM of glycine adjusted to pH 8.3 and 0.1% SDS). The separated protein was blotted on a PVDF membrane which was prewetted in 100% methanol and then equilibrated through protein transfer buffer, using an electro blotter at 100 mA current all over the night. Subsequently, the membrane was incubated in other solution called blocking solution which contains 5% nonfat dry milk prepared in TBST buffer (10 mM of tris, pH 7.5, 500 mM of Nacl, 0.05% Tween-20) for 1 h at 40 rpm at 25°C temperature. The membrane was washed thrice with TBST buffer for 5 min each. In the next step, blot was incubated in the same buffer containing 1% nonfat dry milk and 1:300 dilution of antirabbit antisera of choline oxidase (procured from professor Murata) for 1 h at room temperature and at 40 rpm in the shaker. The blot was again washed thrice with TBST buffer for five, 5 min and then incubated with secondary antibodies (antirabbitIgG, conjugated with horseradish peroxidase) for 1 h. In the last, the membrane was incubated with DAB (diaminobenzidine, 1.6 mgl^{-1} prepared in TBS) till the color developed.

Evaluation of Transgenic Plants for Stress Tolerance

Leaf disc assay for sensitivity against salinity stress

Leaves from healthy and mature transgenic and control plants were detached and washed thoroughly with sterile distilled water. Discs of around 1.0 cm in diameter were punched out by the help of punching machine from these leaves. Then put the discs on different concentrations of Nacl (0-500 mM) for 3-4 days for evaluating their tolerance toward salinity (Fan *et al.*, 1997). These experiments were carried out under continuous florescent white light of 50 µmol $m^{-2} s^{-1}$ at temperature 25 ± 2°C the consequences of various treatments on leaf discs were observed by keeping an eye on phenotypic changes of leaves.

RESULTS AND DISCUSSION

Transformation

In 5 different experiments, a total of 280 cotyledonary node explants were inoculated with *A. tumefaciens*.

The strain used was EHA101 (pGAH/*codA*). A total of 170 green shoots were regenerated on medium supplemented with kanamycin in 6 weeks' time. The regenerated shoots (2-3 cm in length) were cultured in a second round selection medium at the rooting stage. Out of 170 green shoots, 86 shoots directly developed roots on rooting medium (B5 + 2.5 μM IBA) in the presence of kanamycin (12.5 mg/l). The second round of selection during rooting stage effectively remove the nontransformed shoots that may falsly survived on regeneration medium. Of these, 40 healthy plantlets were shifted to soil, and out of them, 25 plants survived. These plants were morphologically similar to seed-raised plants. Around 8-10 weeks were requisite from the starting of cultures up to establishment of transgenic plants in soil (Table 1).

The putative transformants were analyzed by PCR, dot-blot, Elisa and western analysis to confirm the integration and expression of transgenes in transformants.

PCR Analysis

PCR analysis (Figure 2a and b) showed amplification of 0.84 and 0.54 kb fragments corresponding to the coding regions of *hpt, nptII* genes, respectively, demonstrating the presence of transgene in 11 plants.

Dot-blot Analysis

The 11 PCR positive plants were further checked by dot-blot using the probes of both *hpt* and *nptII* genes. Out of them 11 plants, 10 showed hybridization signals (dots) on the X-ray film confirming the integration of both the genes (*nptII* and *hpt*) into the genome of transgenic plants (Figure 3). Since *nptII* and *hpt* genes flank the *codA* gene on the T-DNA thus the entire T-DNA might have integrated into the genome.

ELISA

Total protein extracted from the transformed and untransformed plants was subjects to ELISA test to

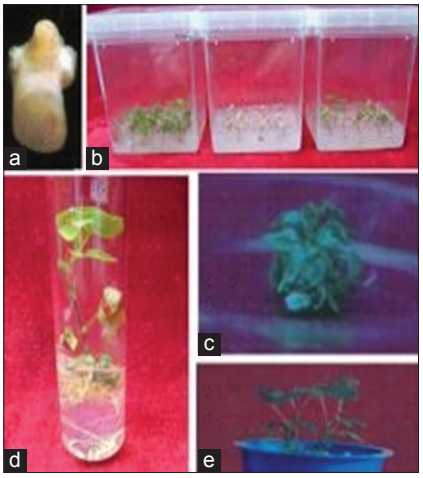

Figure 1: (a) Cotyledonary node explant, (b) selection of explant on kanamycin and hygromycin, (c) putative transgenic shoot, (d) shoot on rooting medium, (e) putative transgenic plant in pot

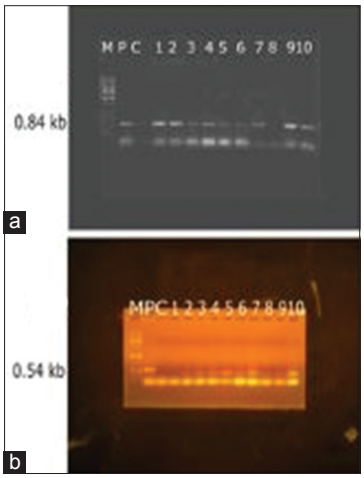

Figure 2: (a) Amplification of 0.84 kb fragment corresponding to the coding regions of *hpt* gene, (b) Amplification of 0.54 kb fragment corresponding to the coding regions of *nptII* gene.

Table 1: Summary of genetic transformation of 16-h old cotyledonary node explants with *Agrobacterium tumefaceins* strain EHA101 harboring a binary vector pGAH/*codA*

Experiments	Total number of explants inoculated with *Agrobacterium*	Number of shoots regenerated on selection medium*	Number of shoots rooted as selection medium**	Number of putative transgenic plant established in soil	Plants positive for *nptII* and *hpt* by PCR	Plants positive for both the *nptII* and *hpt* genes by dot-blot	Plants positive for choline oxidase by Western analysis
1	60	34	18	40 (25)	25 (11)	10 (10)	2 (2)
2	60	41	19				
3	72	40	22				
4	48	30	15				
5	40	25	12				
Total	280	170	86	40 (25)	25 (11)	10 (10)	2 (2)

*Selection medium for shoot regeneration B_5+BAP (1.0 μM)+kanamycin (75 mg/l)+cefotaxime (500 mg/l), **selection medium for root induction B_5+IBA (2.5 μM)+kenamycin (12.5 mg/l)+cefotaxime (350 mg/l), ( ) values in parenthesis indicate the number of plants analyzed. PCR: Polymerase chain reaction

determine the presence of choline oxidase using polyclonal antibodies raised against this protein. The protein sample from transformed plants gave color reaction with the substrate P-nitrophenylphosphate (pNPP) while protein from untransformed plants did not give any color reaction, other negative controls such as only antigen, antigen + primary antibody, antigen + substrate, primary antibodies + buffer and only buffer did not give any color reaction.

Western Blotting

The presence of choline oxidase in transgenic plants was evidenced by SDS-PAGE and western blotting (Figure 4). The total proteins extracted from transgenic and control plants were immunoblotted and checked using antibodies specific to choline oxidase. The PCR and dot-blot positive plants analyzed by Western blotting showed the signals of the 65 kDa protein which corresponds to choline oxidase. No such band was observed in nontransformed (control) plants. In addition, a band at around 70 kDa region was detected as noted earlier in *Arabidopsis* (Hayashi *et al*., 1997) and rice (Mohanty *et al*., 2002). The 70 kDa band could be a precursor of choline oxidase containing a transit peptide. The result analyzed the presence of a precursor protein having a transit peptide that was targeted to chloroplast and processed accurately. These results convincingly demonstrated that the transgene was actively transcribed and translated.

Leaf Disc Assay for Sensitivity against Salt Stress

Leaf disc senescence test was bring about to test the sensitivity of transformed plants toward salt stress. Leaf discs of the diameter (1 cm) from the transgenic and nontransgenic (control) lines were floated on NaCl (50-200 mM) for 4 days (Figure 5). The leaf disc from nontransgenic plants showed complete senescence (Figure 5a) and those from transgenic plants remained green (Figure 5b).

Thus, these experiments prove the potential of *Agrobacterium* - mediated transformation using cotyledonary node explants of mung bean. This transformation method has been based on direct shoot regeneration from cotyledonary node (without cotyledons). This study signifies the role of GB-biosynthetic pathway as accomplished by *codA* gene encoding for choline oxidase for improving stress tolerance in mung bean plants which are nonaccumulator of it. Such transgenic systems provide helpful models for the discretion use of GB against abiotic stress conditions.

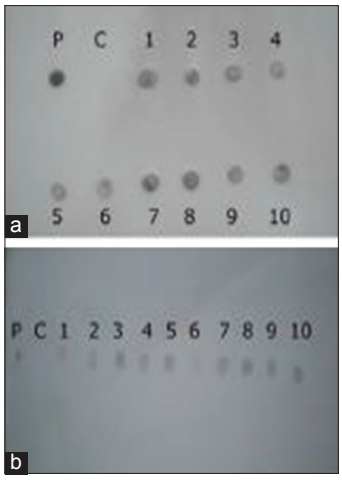

Figure 3: (a) Hybridization signals (dots) on the X-ray film confirming the integration of *nptII* gene., (b) Hybridization signals (dots) on the X-ray film confirming the integration of *hpt* gene.

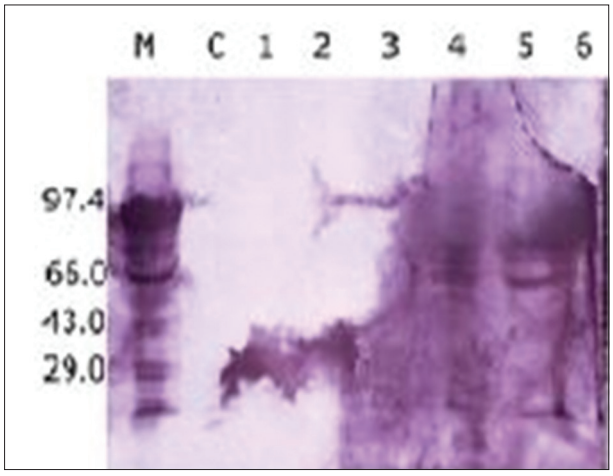

Figure 4: Western blotting showing signals of 65 kDa protein corresponding to choline oxidase

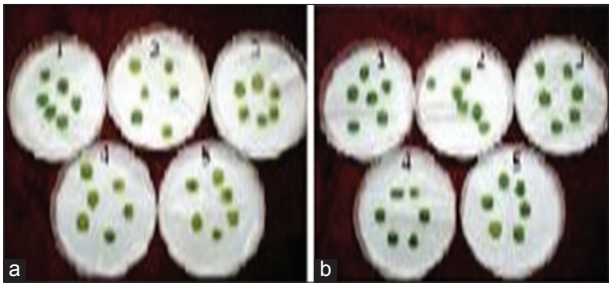

Figure 5: (a) leaf disc from non-transgenic plants showing complete senescence (b) leaf disc from transgenic plants

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