RESEARCH ARTICLE

DEVELOPMENT OF SUGARCANE PLASTID TRANSFORMATION SYSTEM USING PARTICLE BOMBARDMENT

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Received-30.03.2017, Revised-01.05.2017

Abstract: Chloroplast transformation has number of advantages over nuclear transformation like high-level of transgene expression, transgene containment and lack of gene silencing. The present work carried out to develop a chloroplast transformation protocol for sugarcane. Embryogenic calli of sugarcane variety Co86032 used as target tissue for transformation. Chloroplast specific transformation vector pZE39 having *NPTII* and *GFP* genes flaked by *trnG* and *pzbZ* of chloroplast sequence used for transformation. Selection of transformants were carried out at callus, shoot and rooting stages with Geneticin ranging from 25 to 75 mg/l during different selection cycles. Most of the regenerated shoots turned albino during selection. Among different bombardment parameters tested, rupture discs pressure at 1350 psi, distance between target tissue and stopping screen at 8 cm and expose of target tissue to light for 8 days before bombardment found prominent in producing more number of green and resistant plants on selection medium. Molecular analysis revealed that out of 146 plants tested, 44 plants are found PCR positive. Four of eleven PCR poitive plants showed positive by Southern hybridization and five of ten plants are showed positive signals for GFP. This is the first report on an attempt to develop a chloroplast transformation protocol for sugarcane.

Keywords: Chloroplast transformation, Co86032, NPTII, GFP, Particle bombardment

INTRODUCTION

The modern sugarcane cultivars were developed from initial hybrids having chromosome number (2n) ranging between 100 and 130 as a result of hybridization and repeated clonal selection (Christy *et al*., 2009). Though classical plant breeding has been main approach for sugarcane development, various problems associated with sugarcane development are; complexity of the sugarcane genome, it's narrow genetic base, high polyploidy and heterozygocity, poor fertility and time required for a new variety to reach commercialization (Lima *et al*., 2002; Christy *et al*., 2009).Biotechnology offers an alternative and excellent opportunity for sugarcane crop improvement. But it also has certain constrains such as transgene inactivation, somaclonal variation, low transformation efficiency and long time required for regeneration and commercialization. Tissue culture forms a major part of biotechnological technique. The advantages of tissue culture methods are aid in the mutation, propagation and breeding study, production of virus free plants of sugarcane (Nickell 1967 and Leu 1972).

Different transformation techniques like Electroporation (Rathus and Birch 1992), Polyethyleneglycol treatment (Chen *et al*., 1987), Particle bombardment (Franks and Birch 1991), and *Agrobacterium* mediated transformation (Arencibia *et al*., 1998) were used for sugarcane transformation. Transformation of sugarcane for various traits like resistance to lesser cornstalk bore (Nutt *et al*., 1999), increased total sucrose concentration (Wu and Birch 2007), transformation with Cry1Aa3 for resistanct to early shoot borer (Kalumke et al, 2009), *cry1Ab* and aprotinin genes for resistant to early shoot borer (Arvinth *et al*., 2010) and salt tolerance (Rani *et al*., 2012) has been carried out. The most regular method of transformation used is nuclear transformation have various concerns like low expression levels, inconsistent expression profile among transgenic population and transgene flow (Aziz *et al*., 2005) Plastid or chloroplast transformation is an alternative method to overcome problems related with nuclear transformation. Chloroplast in plants also known as plastid, carry out photosynthesis as well as other important activities like evolution of oxygen, amino acid and fatty acid synthesis, sequestration of carbon, and starch production (Wani *et al*., 2010). As plastid transformation has a number of merits over nuclear transformation like high-level transgene expression, multi-gene engineering, transgene containment and lack of gene silencing and position effect, (Lee *et al*., 2006) this approach effectively used for genetic improvement of various plants. Plastid transformation of different crops like carrot, alfalfa, Brassicacea, cotton, soybean, tobacco, rice, tomato, egg plant, cabbage have been carried out for various agronomic traits and also to produce therapeutic proteins (Cosa *et al*., 2001; Skarjinskaia *et al*., 2003;

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Dufourmantel *et al*., 2004; Wurbs *et al*., 2007; Singh *et al*., and Wei *et al*., 2011). Many pharmaceutical proteins like next-generation antibiotics, antibody fragments, blood coagulation factors also expressed in plastid successfully (Oey *et al.*, 2009; Verma *et al.*, 2010; Gisby *et al.*, 2011 and Lentz et *al.*, 2012). Khan and Maliga developed a marker system for chloroplast transformation to differentiate between individual transformed and wild type plastids which facilitate the extension of plastid transformation to non-green plastids in embryogenic cells of cereal crops. There are no published reports on genetic engineering system for sugarcane chloroplast.

In present work we have tried to develop a chloroplast transformation system for sugarcane using a chloroplast specific transformation vector pZE39 that targets gene at *trnG* and *psbZ* region of chloroplast genome. The confirmation of transgene was done by PCR, Southern blot hybridization and GFP analysis. This system will be useful in transfer of gene in sugarcane for important traits like stress resistance, valuable products production and can be use effectively in developing elite variety of sugarcane.

MATERIAL AND METHOD

Plant materials

Sugarcane variety Co86032, a commercially released and popular in state of Maharashtra, India, was selected for chloroplast transformation. Leaf segments of 6-8 cm below the apical dome was selected from 6-8 months old field grown sugarcane. These segments were sterilized with ethanol (70%) for 1-2 min and then with $HgCl_2$ (0.1%) for 10 min followed by thrice washing with sterile distilled water. Outer layers were removed aseptically and leaf segments of ~2 mm thick were inoculated on MS salts and vitamins medium (Murashige and Skoog 1962) supplemented with 3 mg/l 2, 4-D, 0.5 mg/l Kinetin, 500 mg/l Proline and 3 % Maltose (3MES) for callus induction and proliferation. Cultures were incubated in dark at 26 $\pm$ 2 ^{o}C. Embryogenic calli developed after 6-8 weeks were taken for transformation experiments.

Transformation vector

The plasmid transformation vector pZE39 was constructed by placing the expression cassettes containing the green fluorescent protein gene (*GFP*) and the neomycin phosphotransferase II gene (*NPTII*) (Fig.1). This cassette is flanked by trnG and psbZ sequences of sugarcane chloroplast genome and kindly provided by Dr. Ralph Bock, Max Planck Institute, Germany, used in transformation experiments. This vector consists of *gfp* as the reporter gene derived by *CrPrrn-G10L* promoter followed by terminator *Cr.3atpA* and the selectable marker gene *nptII* with *Nt.PrrnG10-L* promoter and *Nt.TrbcL* terminator. The entire vector pZE39 transfrred into *E. coli* (DH5α) for isolation of plasmid DNA to use in transformation experiments.

Gene coating and Plastid transformation parameters

About 10 µl plasmid DNA (1 µg/µl) of pZE39 was mixed with 50 µl gold suspension. While agitating this suspension on vortex mixer, 50 µl of $CaCl_2$ (0.25 mM) followed by 20 µl of Spermidin (100 mM) were added and vigourously agetated the suspension for 30 min at 4 ^{o}C in cold room. After 30 min incubation, suspension was centrifuged for few seconds and discarded the supernatant. The pellet was washed thrice with 100% ethanol and finally dissolved in 36 µl of absolute alcohol that served for 6 bombardments.

Embryogenic calli of Co86032 was used as target tissue for bombardment placed on osmotic medium, 3MES supplemented with 0.2 M each of sorbitol and mannitol for osmotic treatment to the callus for 4 h prior to bombardment. Embryogenic calli of 2-3 mm diameter were arranged in a circle of 3 cm diameter in 9 cm petri dishes on osmotic medium [Fig 2]. These calli were bombarded with 0.6 mm diameter gold particles coated with plasmid DNA of chloroplast specific transformation vector pZE39. Bombardment was carried out using the PDS 1000/He biolistic system (Bio-Rad, Richmond, USA). Different parameters like rupture disc pressures of 1100, 1350 and 1550 psi, distance between rupture disc and target tissue for bombardment at 6 and 8 cm and expose of calli to light before taking for bombardment for 3, 4, 5, and 8 days were tested.

Selection and regeneration of transformants

The bombarded calli were incubated in dark on the same medium for overnight and then next day transferred to 3MES for next 5 days to recover from bombardment shock. After 5 days, they were transferred to 3MES with geneticin 25 mg/l for selection of transformed cels. These calli were subcultured at 15 days intervals each for 3-4 times and freshly grown geneticin resistance calli were transferred to 3MES medium with higher concentration of geneticin (50 mg/l) for 2-3 rounds selection. Geneticin resistant actively proliferating calli transferred to regeneration medium, basal MS medium supplemented with kinetin (0.5 mg/l), NAA (0.5 mg/l), geneticin (50 mg/l) [SRM] and incubated at 26 $\pm$ 2 °C with 16 h light and 8 h dark photoperiodism for 3 cycles of 21 days interval. The green shoots at height of 7-10 cm were excised and transferred to rooting medium (MS + 0.5 mg/l NAA + 40 mg/l geneticin). Juvenile rooted plants that were survived on selection medium were transferred to soil pots containing sterilized mixture of soil, sand manure (1:1:1) and grown in greenhouse for further growth of the plant.

Confirmation of transformants

PCR analysis

Sugarcane DNA was extracted from the isolated chloroplasts of both the transformed and untransformed plants for PCR analysis as per method described by Aljanabi (1999). PCR amplification was done using gfp specific primers; forward: 5'- CGT AAGGGGAAGG GGAAAAC-3' and reverse: 5'- CCATGTGTA ATCCCAGCAGC- 3' to obtain 886 bp PCR amplicon. PCR conditions were worked out using following conditions for *gfp* gene primers for pZE39 vector. One cycle of initial denaturation at 94 °C for 5 min followed by 35 cycles: 94 °C for 30 sec, 56 °C for 30 sec, and 72 °C for 45 sec, and final extension at 72 °C for 10 min. Finally the reaction was held at 4 °C till reaction terminates. PCR product was run on 1% agarose gel for observation of bands.

Southern blot hybridization analysis

Genomic and chloroplast DNA of the PCR positive putative transformants was extracted to perform Southern blot hybridization for identification of integratin and copy number of the transgene. Ten µg of DNA was digested with restriction enzyme XhoI at 1.5 U/µg DNA concentration (37 °C, overnight) and applied to 0.8% (w/v) agarose gel and electophoresed at 15 V for 12 h. The separated DNA was transferred to positively-charged Nylon Membrane (PALL,USA) overnight and cross-linked with UV at 1200 jouls for 90s. GFP gene specific PCR product of size 886 bp used as probe labeled with (^{32}P)-dCTP (BRIT, India) using Random primer DNA labeling kit and used for hybridization. The DNA-fixed membrane was hybridized using ULTRAHyb Hybridization Buffer (Ambion, USA) at 42 °C for 16 h with the probe in a hybridization oven. Blots were washed and exposed to X-ray film (Fuji X-ray film Super HR-U, 16.5 cm x 21.6 cm). These X-ray film cassettes were incubated at -70 °C for 48 h to develop autoradiogram. These exposed X-ray films were developed and photographed for recording the bands.

GFP expression detection

Confocal microscopy was used to determine the expression of GFP protein in chloroplast of transplastomic plants. Subcellular localization of GFP was verified by laser scanning confocal microscopy (PALM Robo Axio Observer D1, Zeiss make) at IISER, Pune. The system utilizes excitation at 395 nm, and emission at 509 nm. The sterile transgenic leaves of different lines were collected and a transverse section mounted on slide. In this study, the strong signal of green fluorescence emission from transformed cells was observed.

RESULT

Development of embryogenic calli and optimization of antibiotic concentration for selection of transformants

MS medium containing 2,4-Dichloro phenoxyacetic acid (3 mg/l), maltose (3%), kinetin (2 mg/l), proline (500 mg/l) induced about 80% of embryogenic callus (data not shown). Embryogenic calli for transformation experiment developed on media as described Wadyalkar et al (2011). Antibiotic sensitivity test for Geneticin on embryogenic calli was performed. Geneticin 25 mg/l showed that most of the callus was fresh and very little browning was observed. Browning was started increasing from 50 mg/l to 75 mg/l and complete browning was found in 100 mg/l. Hence, geneticin at the concentration of 50 mg/l where it inhibited 60-70% of the callus growth was selected for transformantion.

Before hardening stage, selected plants were transferred to lower dose of selection (40 mg/l) for gradual removal of antibiotic pressure for further acclimatization of regenerated plants. Shoots with 3-4 cm height were excised and successfully rooted on MS media supplemented with 0.5 mg/l NAA with selection agent geneticin at 40 mg/l concentration. The survival percentage of regenerated plantlets on rooting medium was 30%, after transfer to soil pots was 27% where as survival rate of plants in green house was 40%.

Optimization of particle bombardment for chloroplast transformation

Embryogenic calli developed from leaf roll discs was used in transformation experiments. Effect of light on callus prior to transformation was studied. Embryogenic calli was exposed to fluorescent cool light for 3, 4, 5, and 8 days before taking for bombardment. The embryogenic calli exposed to light for different days was arranged on osmotic medium in plats were bombarded with *gfp* & *nptII* gene construct. Different bombardment parameters tested are distance between rupture disc and target tissue and pressure of rupture discs. After bombardment, bombarded calli was transferred on osmotic free medium and incubated for 5 days to recover from bombardment shock. Bombarded calli were exposed to selection of transformants for 3-4 rounds of selection pressure. Each selection round of three weeks on callus proliferation medium with 50 mg/l Geneticin. Selection of transformed calli was followed by regeneration of shoots and roots with selection. A total of 1476 plants were regenerated after bombardment on continuous selection pressure. However, most of the plants (1027) that were regenerated were turned into albinos.

In the present experiments, effect of light on callus prior to transformation was studied. Embryogenic calli was exposed to fluorescent cool light at various days were 3, 4, 5, 7 and 8 days. Callus exposure to light might have advantage in converting proplast to chloroplast hence more number of green plants (49) were regenerated at 8 days light incubation as compare to 3 days (7 plants).

After transformation for recovery, calli transformed to medium without selection for 7 days followed by further transfer of calli on selection medium having geneticin at concentration 25 mg/l with further

selection at increasing concentration to 50 mg/l and 75 mg/l in subsequent selection cycles.

In the present investigation, rupture disc pressure at 1350 psi was found more beneficial as compare to 1100 and 1550 psi. Around 50 plants were regenerated under continuous selection at this pressure. It appears that 1100 psi is less effective in transferring the particles and 1550 psi is high pressure that might damage the tissue. Distance between stopping screen (SS) to target tissue (TT) also has correlation. Lower the pressure (1100 psi) and shorter the distance (6 cm) yielded more number of green plants (5) as compared to 8 cm (3 plants). This pressure might not be enough to penetrate the particles. However, at the higher pressure (1350) shorter the distance has yielded less number of green plants (4) as compared to longer distance of 8 cm (49 plants). Higher pressure and shorter the distance might have deleterious effect on the cells.

Confirmation of transgene integration and GFP detection

Integration of transgenes confirmed with the help of gene specific PCR producing 926 bp for *nptII* and 886 bp for *gfp* products (Fig. 3 A and B). Out of total 146 plants tested, 44 plants are found PCR positive. Southern blot hybridization analysis was performed using total genomic DNA isolated from transformed and untransformed sugarcane plants. Digestion of total DNA carried with XhoI restriction enzyme (Fig. 3 C). DNA digested by XhoI was analyzed by Southern blot using an 886 bp probe resulting in a hybridization signals of size 1.5 kb for four transplastomic lines indicating integration of gene (Fig. 3 D). Four out of eleven PCR confirmed plants showed positive signals for Southern blot hybridization. Plastid based GFP accumulation was analysed in five out of ten lines after observation under confocal laser scanning microscopy. A distinct plastid localized-GFP fluorescence observed in leaf section under confocal laser scanning microscope (Fig. 4).

In the present study, chloroplast specific transformation vector pZE39 which contain as cassette with *NPTII* and *GFP* gene flanked by trnG and psbZ chloroplast specific sequences was used. After several round of selection well survived plants processed for further and transfer to green house. DNA isolated from plants growing in green house for analyses by PCR using gene specific primers for GFP. After PCR analysis, 13 out of 35 clones showed PCR amplification. The eleven PCR positive plants were subjected to further confirmation by Southern hybridization. A GFP specific probe of 886 bp labeled with ^{32}P used in hybridization process. Four out of eleven plants showed signals for southern hybridization. While in gfp protein detection study 5 out of ten PCR positive plants showed presence of GFP signals in plants.

DISCUSSION

The present work carried out in terms to develop a suitable chloroplast transformation protocol for sugarcane. This is the first report on chloroplast transformation in sugarcane. The optimization of antibiotic concentration for transformants selection has been carried out for geneticin antibiotic. Among the various concentrations tested 50 mg/l geneticin found effective at initial stage of selection at calli level. While it is increased further upto 75 mg/l for selection of regenerated plants. The geneticin concentration at 45 mg/l for transformants selection has been used (Bower and Birch, 1992).

Transformation efficiency observed low with other chloroplast transformation vector with flaking sequences from tobacco or Arabidopsis used for potato, tomato and *Lesquerella* (Sidorov *et al*., 1992; Skarjinskaia *et al*., 2003 and Ruf *et al*., 2007). The vector used in current research work contains flanking sequence trnG and psbZ from sugarcane genome itself, which helps to reduced chance of low transformation efficiency. It was reported that in carrot transformation use of carrot chloroplast species-specific vector showed higher transformation efficiency (Kumar *et al*., 2004). In case of solanaceous crop chloroplast transformation hinder by use of non-green tissue (Bogorad, 2000). There are number of proplastid structure present in non-green tissues which have different gene regulation mechanism than mature chloroplast. Upon transformation transformed proplastid has to develop into mature chloroplasts and transformants has to survive on selection.

In our study effect of light on callus prior to transformation was also studied. Embryogenic calli was exposed to fluorescent cool light at various days were 3, 4, 5, 7 and 8 days. Callus exposure to light might have advantage in converting proplast to chloroplast hence more number of green plants (49) were regenerated at 8 days light incubation as compare to 3 days (7 plants).

However it was reported that development of dark-grown tobacco suspension cell model can be used to investigate the transformation potential of undeveloped plastids (Langbecker *et al*.,2004). Results indicate that the rate-limiting steps for nuclear and plastid transformation are different and must be optimized separately and it also indicated that plastid size, subcellular localization and developmental stage are apparently not the rate limiting factors for successful and efficient plastid transformation.

After transformation for recovery, calli transformed to medium without selection for 7 days followed by further transfer of calli on selection medium having geneticin at concentration 25 mg/l with further selection at increasing concentration to 50 mg/l and 75 mg/l in subsequent selection cycles. Callus proliferation for four days after bombardment

followed by 4-6 subculture of 2 weeks on selection medium produce more number of transformed plants. Callus proliferation for four days after bombardment followed by 4-6 subculture of 2 weeks on selection medium produce more number of transformed plants (Bower and Birch, 1992).

Application of chloroplast transformation system in crop like sugarcane would be a most advantages in its development programme. With using parameters mentioned, sugarcane transformation for particular trait can be carried out in future. With lot of advancement in chloroplast transformation technique it is still need to develop a stable and more effective system for agronomically important crops.

ACKNOWLEDGEMENT

We are grateful to the Department of Biotechnology (GOI), New Delhi for providing financial assistance to this work through project (No.BT/PR13389/NDB/53/28/2009) and to Dr. Ralph Bock, Max Planck Institute, Germany for providing chloroplast transformation vector.

Table 1. Details of bombardments using different parameters for embryogenic calli and regenerating tiny shoot buds

Days light incubation (Prior to bombardment)	Rupture disc pressure (psi)					
	1100		1350		1550	
	Distance between Stopping Screen to Target Tissue (cm)					
	6	8	6	8	6	8
3	22	17	54	64	6	6
4	NT	60	NT	107	NT	NT
5	6	6	37	37	6	6
7	6	6	21	21	5	5
8	18	26	10	122	NT	NT

NT= Not tested

Table 2. Number of plants regenerated using different parameters from embryogenic calli and regenerating tiny shoot buds

Days light incubation (Prior to bombardment)	Rupture disc pressure (psi)					
	1100		1350		1550	
	Distance between Stopping Screen to Target Tissue (cm)					
	6	8	6	8	6	8
3	0	0	4	3	0	0
4	NT	0	NT	0	NT	NT
5	0	0	0	0	1	0
7	0	0	0	8	0	0
8	5	3	0	35	NT	NT

NT= Not tested

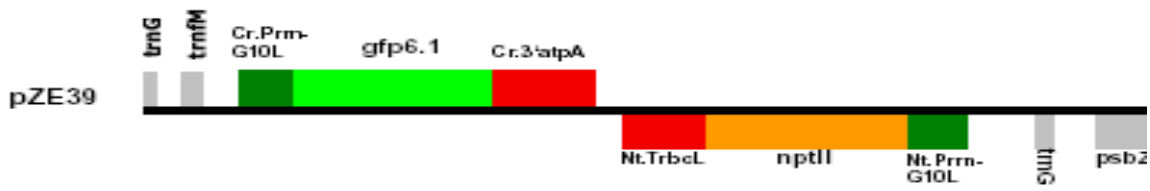

Fig.1

Fig. 1 Physical map of chloroplast specific transformation vector pZE39

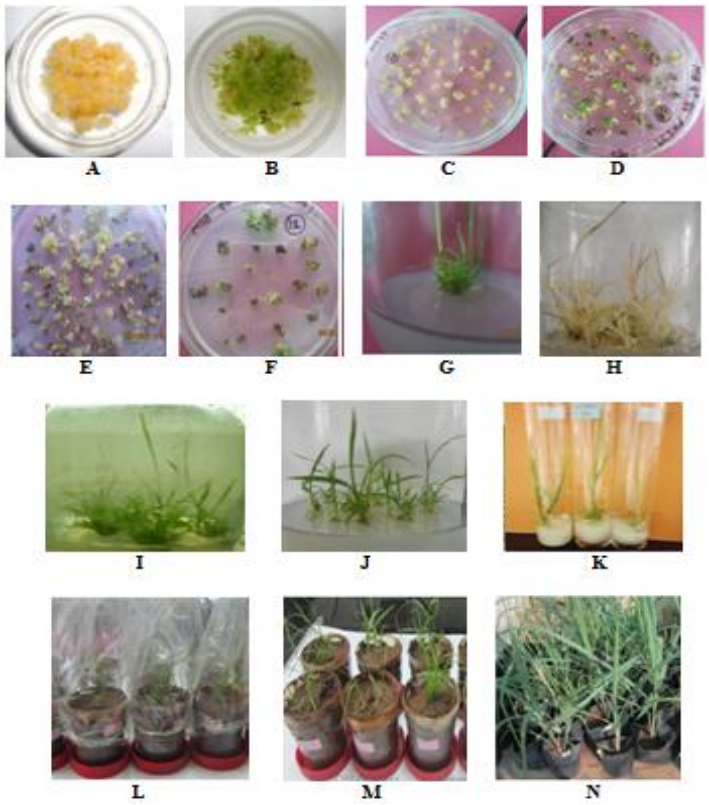

Fig. 2

Fig. 2 Stages of selection of chloroplast transformed materials from callus to the regenerated plantlets & hardening of putative transformed plants. **A** & B –Callus and tiny regenerating shoot buds on osmotic medium used for bombardment**,** C & D - Bombarded callus and tiny regenerating shoot buds on osmotic free medium for one week**,** E - Bombarded callus on 3 cycles of selection (50mg/l geneticin)**,** F - Tiny regenerating shoot buds on 2 cycles of selection (50mg/l geneticin)**,** G –Regeneration and plantlets growth on $4^{th}/3^{rd}$ selection cycles (75 mg/l geneticin), H – Albino plants produced during selection. I- Resistant plants on shooting media, J- Resistant plants separated from bunch and put on solid rooting medium, K- Resistance plants separated from bunch and put on liquid rooting medium L-Resistance plants survived on selection medium transferred to soil pots covered with polypropylene bags, M- Plants in soil pots for hardening, N- Hardened antibiotic resistance plants in green house.

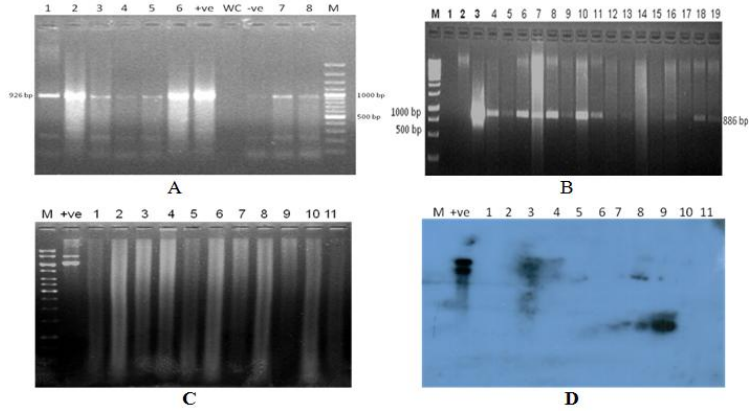

Fig. 3

Fig. 3 PCR and Southern-blot analysis of putative transplastomic plants. A- PCR using *Npt*II gene specific primers 1 to 8 are plant numbers; +ve = positive control plasmid DNA; M- ladder 100bp; WC-Water Control, -ve = control plant DNA, B- PCR analysis using *gfp* gene specific primers. M= 1 kb ladder, 1= water control, 2= untransformed plant DNA, 3= plasmid pZE39, and 4 to 19 = chloroplast transformed plants DNA, C- transgenic plant DNA digested with XhoI, D- Southern blot hybridized with GFP probe, M- 1 kb marker, +ve- Plasmid as positive control, 1 to 11- PCR positive transgenic plants derived after repetitive subculture in liquid medium under Genetcin selection.

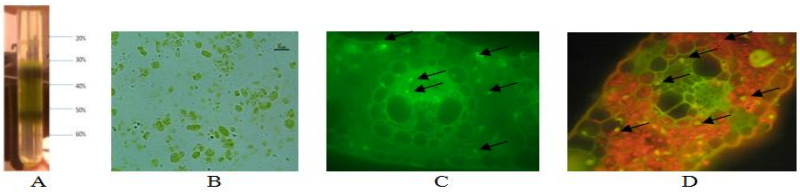

Fig. 4

Fig. 4. Chloroplast isolation and localization of GFP protein in leaf sections. A- speprated chloroplast layer in sucrose gradient (20-60%), B- Isoalted chloroplast under microscope (400X). was observed from transplastomic line. C- The emission of green fluorescence excited by a 395 nm laser, D- The merged image is of chlorophyll and GFP fluorescence (marked with arrows).

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