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SOMATIC EMBRYOGENESIS FOR *AGROBACTERIUM* MEDIATED TRANSFORMATION OF TOMATO- *SOLANUM LYCOPERSICUM* L.

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Abstract *Agrobacterium* mediated transformation through somatic embryogenesis was achieved using cotyledon explants of tomato cv. Shalimar (*Solanum lycopersicum* L.). Somatic embryos were induced directly from the cotyledon explants on MS medium supplemented with 2, 4-D (5mg/l) for the first time, and also through the callus produced from cotyledon explants on MS liquid medium supplemented with 2, 4-D (2mg/l). The maturation of somatic embryo globular to torpedo was presumed by using sorbitol (0.4 M) and ABA (0.25mg/l). The competency of induced somatic embryos for agrobacterium mediated transformation was confirmed by using GUS reporter gene in pBI121 vector and the npt II with PCR. The transformant was selected using kanamycin (100mg/l). Seventy percentage of transformation efficiency was obtained from the transformed somatic embryo.

Keywords: pB1121, Shoot regeneration, transformation, 2, 4-D

Introduction

Tomato is an important worldwide commercial crop. World tomato trade is growing rapidly [25]. Several transgenic traits encoding genes were introduced in to tomato to improve their nutritional quality a path started by Calgene Inc.[14], all these studies were carried out by adopting different regeneration methodology standardised henceforth. Micro propagation through somatic embryogenesis provides an efficient mean of producing large numbers of elite or transgenic plants. The potential use of somatic embryogenesis in developmental studies, crop improvement and genetic transformation has been widely recognized and the number of species displaying the potential for somatic embryogenesis is constantly increasing [9]. Somatic embryogenesis can probably be achieved for all plant species provided that the appropriate explant, culture media and environmental conditions are employed [2]. Somatic embryogenesis in tomato is still at infancy, and substantial research is still required to be undertaken to obtain high quality somatic embryos, this technique will be usefull for crop improvement using *in vitro* culture and transformation [3].

Agrobacterium mediated transformation have been used as an effective tool for transfer of gene into plant system, while use of somatic embryos as explant in transformation of plants is a recent approach. Somatic embryos consist of embryogenic cells when successfully transferred can produce large number of transgenic plant from a single explant. Hence, *Agrobacterium* mediated transformation of somatic embryos hold an important role in transgenic plant production.

Only few works have been reported for Agrobacterium mediated transformation using somatic embryos, in plants like walnut [17], cork oak (*Quercus suber* L.) [22], alfalfa (*Medicago falcata* L.) [23] and *Vitis rotundifolia* [5] so standardising succeful regeneration protocol for agrobacterium mediated transformation using somatic embryo in tomato would help improve the efficiency of obtaining the stable transformant as the gene has been transformed to the embryonic tissue. The present study was aimed to develop plant regeneration system for tomato via somatic embryogenesis and optimizing several key factors to utilize tomato somatic embryos for *Agrobacterium*-mediated genetic transformation using cotyledon explants which not reported earlier.

Experimental Procedure

Collection and germination of seeds

Tomato seeds var. Shalimar was obtained from the Defence Institute of High Altitude Research, Leh, India. Seeds were washed with distilled water for 5 times, followed by treatment with 0.1% $HgCl_2$ for 3 min and rinsed with sterile water 6 times to remove the sterilant. Seeds were germinated in test tubes containing moist, sterile cotton (Fig.1a). Seedlings were grown under white

fluorescent light (40 µmol m^{-2} s^{-1}) at a 16 h-day photoperiod and 25 ± 2ºC.

Somatic embryo Induction, development

For the induction of somatic embryo cotyledons were excised from two-week old seedlings and the whole cotyledons were inoculated onto MS medium. They were aseptically placed with the abaxial surface touching the MS medium + B5 vitamins supplemented with different concentrations of 2, 4-D (0.5-25mg/l), NAA (2-10mg/l) and TDZ (2-8mg/l) separately and in combination.

Maturation of Somatic embryo and plant development

After 2-3 weeks of culture in various described media, the explants along with somatic embryos of heart shaped stage were transferred to maturation medium supplemented with various plant growth regulators such as, BAP (0.5-2.0mg/L), KIN (0.5-2.0mg/L), GA_3 (0.3-1.0mg/L), ABA (0.25/1.0mg/L), MS + B5 vitamins liquid media supplemented with 6% sucrose and MS + B5 vitamins liquid media without sucrose and supplemented with 0.4M sorbitol separately or in combination for studying the regeneration efficiency of somatic embryos developed. The pH of the media was adjusted to 5.8 and media were solidified with 0.2% Phytagel (Sigma, St Louis, MO, USA) and autoclaved for 15min at 121˚C and at a pressure of 1.06Kg cm^{-2}

Bacterial strains and Vector

A. tumefaciens strains LBA 4404 carrying pBI121 plasmid was employed as vector system for transformation. Plasmid pBI121 is a derivative of the binary vector pBin19 and has (1) the NOS promoter *npt*II- NOS terminator (polyA) cassette and (2) the *gus* reporter gene with a portable plant intron (*gus*-int) under the regulatory control of the CaMV 35S promoter and a CaMV-pA sequence at the left border (Fig. 9). For routine use, strains of *Agrobacterium* were grown in the dark at 28°C in agar-solidified Luria Bertani medium supplemented with 100µg/ml kanamycin.

Transformation

The transformation of somatic embryos was carried out following Fachini et al (2008) protocol with modifications. Single bacterial colonies were prepared by serial dilutions, and each colony was then inoculated in liquid LB medium (20 ml) containing 50µg/ml kanamycin. The bacteria were then allowed to grow in the dark at 28°C for 16–18 h at 180 rpm. Bacterial cells corresponding to OD600=0.6 were pelletted by centrifugation (6,000 rpm, 10 min) followed by washing twice with liquid LB. Somatic embryos at torpedo stage were submerged in the bacterial suspension for 20 min, blot-dried on sterile filter paper and finally transferred to MS basal + 0.4M sorbitol. Following co-cultivation, the somatic embryos were washed twice with sterile distilled water, then subjected to two additional rinses with MS liquid medium containing cefotaxime (400µg/ml), blot-dried and transferred to semi-solid MS media containing two bactericidal antibiotic (cefotaxime and carbenicillin, 250µg/ml each). Following 15 days of culture, somatic embryos were transferred to the same medium supplied with one bactericidal antibiotic (cefotaxime or carbenicillin, 400µg/ml) as well as the phytotoxic antibiotic kanamycin (50µg/ml). Following a four week culture period, with subculturing to fresh medium at fifteen -day intervals, kanamycin resistant somatic embryos were transferred to the maturation medium augmented with an elevated level of kanamycin (75µg/ml) and, after 4 weeks, to the germination medium containing 100µg/ml kanamycin for the selection of resistant embryos.

Analysis of putative transformants

Assay of GUS activity

The histochemical assay of GUS activity was carried out as described by Jefferson et al. (1987). Somatic embryos of torpedo stage were soaked in GUS assay buffer in 2 ml microcentrifuge tubes and incubated overnight at 37°C. Plant material was treated at 65°C with 70% ethanol twice at 1-h intervals to remove traces of chlorophyll. The possibility of endogenous GUS expression was tested by subjecting uninfected somatic embryos and tissues to the histochemical GUS assay. GUS expression was visually observed and photographed under a light stereo-zoom microscope (Nikon HF II).

PCR analysis of *npt*II

DNA was isolated from putative transformed and untransformed somatic embryos of torpedo stage. The forward and reverse primer sequences (F – 5'- GAG GCT AAT TCG GCT ATG ACT G - 3' and, R – 5'- ATC GGG AGA GGC GAT ACC GTA – 3' respectively) (Sigma Aldrich, India) used for PCR amplification of the *npt*II gene. The primers were so designed as to give amplification products of the internal sequence of the *npt*II of 1200 bp. The PCR mixture (25 µl) contained 1.5 U *Taq* DNA polymerase, 10 mM Tris-HCl (pH=9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1 µl of each forward and reverse primer at a final concentration of 10 pmol and 50 ng template DNA. 50 pg of pBI121 plasmid DNA was used as a positive control. Samples for enzymatic amplification were subjected to an initial programme of one cycle of 95°C for 150 s, 60°C for 30 s, 72°C for 90 s followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 90 s. Finally, the reaction mixtures were allowed to complete an additional extension of 10 min at 72°C before rapid cooling to 4°C. The PCR was carried out using a Cyber Cycler P series (Peltier model P96+) with the fastest available transitions between temperatures. The amplification products were separated by 1% agarose gel electrophoresis with ethidium bromide solution (0.5µg/ml) in 1× TAE buffer (0.04 M Tris-acetate, 1 mM EDTA, pH=8) at 80 V for 45 min and the gel was photographed under an Alpha Digi Doc System.

Results

Indirect Somatic embryogenesis

embryogenic calli formation (Fig.1c) was observed after two weeks of culture **w**hen the cotyledonary explants from 10 day old *in vitro* germinated tomato plants were cultured in MS media supplemented with 2, 4 -D (0.5-2 mg/l)(Fig.1b). The embryogenic calli when further subcultured for 2 weeks in MS basal liquid medium produced somatic embryos of global stage (Fig.1g). MS media supplemented with 2, 4-D (1.5mg/l) showed maximum percentage of somatic embryogenesis (Tab 1). Whereas the cotyledon explants cultured in MS media supplemented with different concentrations (2 -10 mg/l) of NAA resulted in only callus formation and no embryo formation was obtained in any of the concentration of NAA.

Direct Somatic embryogenesis

Similarly Tomato cotyledon explants cultured on MS media supplemented with 2, 4-D (5 mg/l) showed maximum somatic embryo formation followed by 2,4-D (10mg/l, and 15mg/l) respectively. Direct somatic embryos at globular stage either singly or fused (Fig.1c) heart shaped (Fig.1c) were visible on the surface of swollen cotyledon explants in all the higher concentrations of (5, 10, 15 and 25mg/l) 2, 4-D. After 4 weeks, all the cotyledon explants showed direct somatic embryo formation (Table 1). Somatic embryo formation was not observed after 4 weeks of cotyledon culture on MS basal media, further sub culturing of cotyledon explants on to the same media composition did not show any precipitation of somatic embryos instead green meristematic nodule formation was observed. The cotyledon explants cultured individually in MS media consisting of TDZ, NAA, and the 2, 4-D (0.5 mg/l to 2.5 mg/l) were callused after 3 weeks, and had no visible signs of somatic embryos even after the 4th week. In addition, more than 80% of explants cultured in MS media supplemented with NAA formed roots primarily at the proximal end. Whereas cotyledons cultured on MS media fortified with BAP (2mg/l to 15mg/l) concentrations, were swelled and slightly callused on the cut surfaces after 3 weeks of culture. Sorbitol showed a significant role in the further development of tomato somatic embryos. Somatic embryos were developed in to shoots (Fig.1d and Fig.1e) when maintained in MS + Sorbitol (0.2 to 0.6 M) and the highest rate of germination was in media supplemented with sorbitol (0.4M) (Table.3).

Transformation of somatic embryos

Selection of transformed Somatic embryos

Transient GUS activity was detected by characteristic blue spots which appeared on the somatic embryos 48 h following co-cultivation (Fig.2c). Eventually this blue coloration became uniformly distributed over the entire embryo surface (Fig.2d). No endogenous GUS expression was detectable in non transformed somatic embryos. Putative transformed somatic embryos were selected, based on their growth on media with kanamycin 50µg/ml. The GUS-positive reaction of kanamycin- resistant somatic embryos was unambiguously due to the expression of the transgene *gus* in the tissue and not due to the persisting *Agrobacterium*, used in the transformation experiments. DNA obtained from several independent kanamycin-resistant, GUS-positive lines revealed the specific predicted amplification product of 1.2 kb with *npt*II- gene-specific primers, (Fig.2f). No amplification product was detected in DNA from non transformed shoots.

Transformation efficiency

Somatic embryos of tomato showed 100% transformation efficiency, as all transformed somatic embryos showing GUS activity (Fig.2d), and amplification of npt II marker gene (Fig.2f).Torpedo shape intact somatic embryos on the cotyledon that were directly infected with *Agrobacterium* without pre-culture showed a higher transformation competence than the ones detached from cotyledon and pre cultured prior to bacterial treatment. Transformation efficiency based on GUS expression was 100% without pre-culture, while it varied from 30% to 40% for a 48 hour pre-culture (Tab 4). Elimination of the pre-culture condition was also helpful in producing transformants in kiwifruit and apple [13]. The maximum number of transformation events (approx. 100%) was scored using intact (unwounded) somatic embryos. When somatic embryos were injured manually, a lower number of transformation events (10%) coupled with a high rate of tissue browning were noticed (Tab 4). Thus, wounding was not only unnecessary for inducing transformation but also deleterious to tomato somatic embryos. This was also found to be the case for walnut [17], and tea [18].The transformed embryos germinated in to plantlet in the kanamycin supplemented selection media with proper rotting and morphological character (Fig.2e).

Discussion

The studies on tissue culture of Tomato have been restricted to plant regeneration via organogenesis via leaf and cotyledon explants. Less emphasis has been placed on somatic embryogenesis except from protoplast cultures [4], hypocotyls [20] and from intact seedlings growing on a medium containing high concentrations of cytokinin [8] and [14]. Being a model plant in molecular developmental studies, and important traits has been introduced in tomato somatic embryogenesis in tomato also has considerable importance. It was observed that transformation efficiency of somatic embryos are higher than that of cotyledons or leaf disc thus, may be due to the fact that the whole surface of somatic embryos are exposed to the *Agrobacterium* for infection compared to the exposure of only cut edges in case of former. As the transgenic plant develops from whole transformed somatic embryo, the confirmation of transgene integration at this stage is very relevant and also reduces the time by avoiding occurrence of any false positive transformants. The method utilises a time interval of 4

weeks which is comparable to that of any other explants used for transformation.

Somatic embryogenesis

From our observation, it is evident that the somatic embryogenesis and further regeneration of the embryos can be achieved in two ways, either direct embryogenesis or indirect embryogenesis. In the former the embryos are induced directly from the surface of the cotyledon explant, where as in the latter, an intermediary callus stage precedes the somatic embryo formation. Breaking the dormancy of the torpedo shaped somatic embryos form the threshold in case of many plant species, especially in case of tomato. Also, the fact that regeneration protocols are genotype specific in tomato makes the present study a relevant and significant one, for the researchers who are interested in the genetic modification of tomato. In this study, 2, 4-D (2mg/l) alone did not induce any direct somatic embryogenesis, even though it facilitated somatic embryogenesis after a callus stage. However 2,4-D seems to show negative effect on the further regeneration of shoots from torpedo shaped somatic embryos in tomato, this agrees with the results obtained in carrot [4] and [7] they believed that once the embryogenic potential is induced, the expression of an embryogenic program no longer requires 2,4-D. Other workers have induced direct somatic embryogenesis on Carica [17] and carrot [25]. A recent study of somatic embryogenesis and genetic transformation in Opium [11] showed that in recalcitrant plant varieties, somatic embryogenesis is useful method of transformation. It was showed that sorbitol can be used as a stress agent and carbon source for somatic embryogenesis in *Arabidopsis thaliana* [12].

Microscopic analysis of Somatic embryos

Somatic embryos of globular (Fig. 2a), heart (Fig.2b) and torpedo shape were stained with safranin stain and were clearly visible when observed under microsope 400X magnification.

Conclusion

In conclusion, we have developed culture conditions for somatic embryogenesis from cotyledon in tomato and is found suitable for *Agrobacterium* mediated transformation and showed enhanced percentage of transformation frequency which can be adopted for further transformation studies.

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Table 1- Effect of various types, concentrations and combinations of plant growth regulators on callus induction from cotyledonary segments of tomato on Murashige and Skoog (1962) medium with additives

Hormone level (mg l-1)	Number of Days for callus initiation	Response (%)	Somatic embryo formation	
			Days to formation	Number of Somatic embryos/explants
2,4-D (0.5)	7	65	15	20[b]
2,4-D (1.0)	7	65	15	22[b]
2,4-D(1.5)	7	80	15	30[a]
2,4-D(2.0)	7	95	15	15[b]
NAA (2.0)	10	65	0	0
NAA (5.0)	10	65	0	0
NAA (10.0)	10	65	0	0

Each value is the mean of 3 replications with 20 explants each. Values within a column followed by different letters are significantly different at the 0.05 probability level using DMRT

Table 2- Effect of various levels of 2, 4-D on the direct somatic embryo formation from the cotyledonary explants of tomato (Solanum lycopersicum L.) cv Shalimar

Concentration of 2,4-D (mg/L)	Number of somatic embryos (Mean ± std dev*)	Response In %
5	20.11±10.117a	100
10	16.11 ± 3.756a	90
15	17.12 ± 3.834a	85
20	8.00 ± 3.033b	65
25	5.66 ± 2.422b	65

Each value is the mean of 3 replications with 20 explants each. Values within a column followed by different letters are significantly different at the 0.05 probability level using DMRT.

Table 3- Effect of various Concentration (Molar) of Sorbitol in germination of somatic embryos of tomato (Solanum lycopersicum L.) cv Shalimar

Concentration (M) of Sorbitol in germination medium	Percentage of somatic embryos germinated
0.2	65±5[a]
0.4	75±5[a]
0.6	65±5[a]
0.8	65±5[a]

Each value is the mean of 3 replications with 25 Somatic embryos each. Values within a column followed by same letters are not significantly different at the 0.05 probability level using DMRT.

Table 4- Effect of transformation conditions on the tomato somatic embryo transformation

Method	Transformation efficiency	Number of shoot regenerant/SE	Number of transformed shoots per explants
With pre culture	30 - 40%	1	5[b]
Without pre culture	80 - 100%	1	25[a]
Somatic embry intact with expalnt	80-100%	1	25[a]
Somatic embryo detached from explants	50 – 60%	0	0
Un wounded SE	80-100%	1	25[a]
Wounded SE	30-40%	0	0

Each value is the mean of 3 replications with 20 explants each. Values within a column followed by different letters are significantly different at the 0.05 probability level using DMRT.

Fig. 1

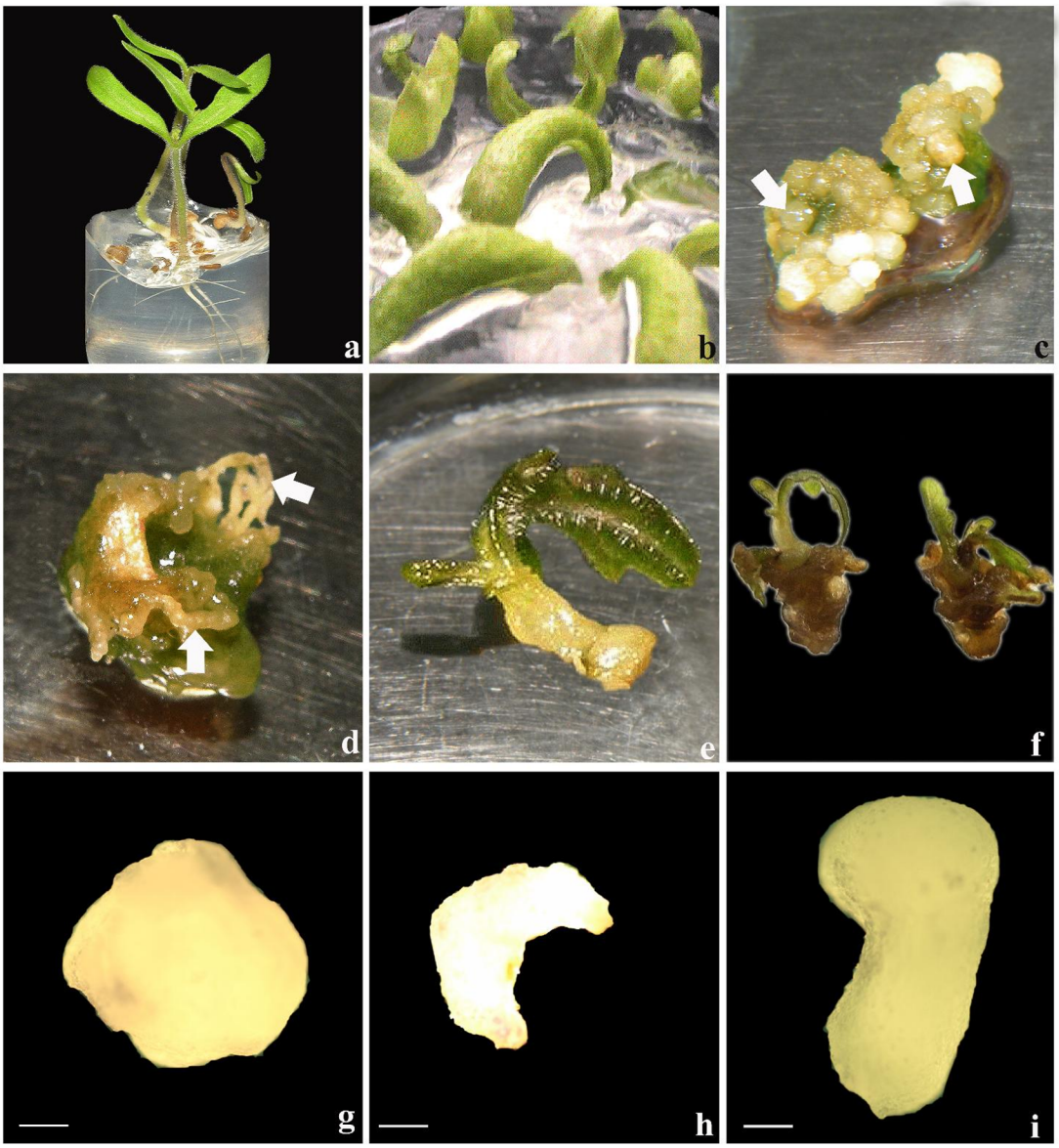

Fig (1a)- Tomato cultivar 'Shalimar' seeds germinated *in vitro* **Fig (1b)** Swollen tomato cotyledons after three days of inoculation in embryogenic media **Fig (1c)** Cotyledon surface showing globular and heart shaped direct somatic embryos (indicated with arrow mark)**Fig (1d)** Cotyledon surface showing torpedo shaped direct somatic embryos **Fig (1e) & (1f)** somatic embryos at cotyledonary stage in the **Fig (1g), (1h) and (1i)** Somatic embryos as observed under microscope with 40x magnification. Scale = 1mm

Fig. 2

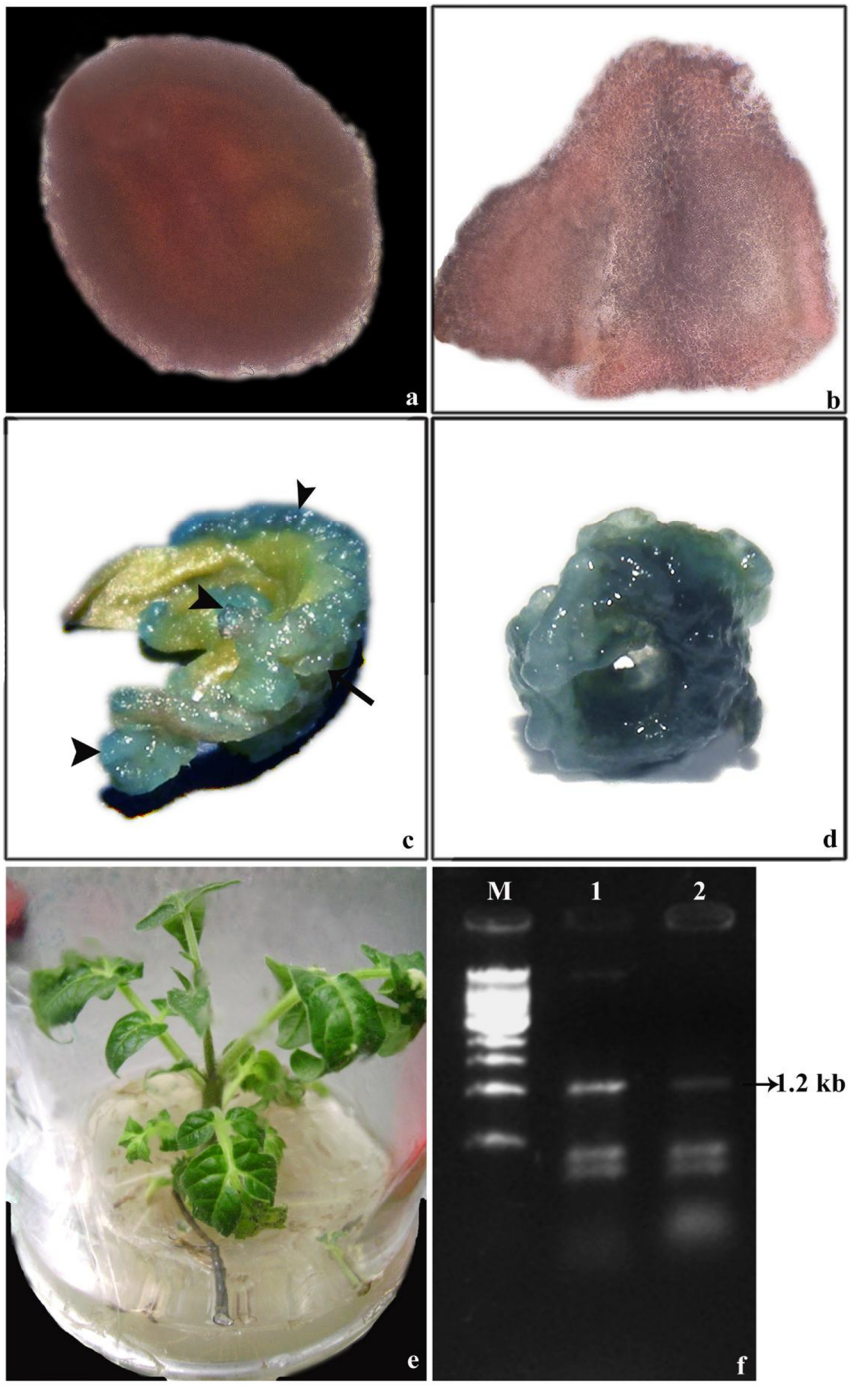

Fig (2a) & (2b)- globular and heart shaped somatic embryos stained with safranin and observed under microscope.**Fig (2c)** Partially GUS stained transformed somatic embryos on the surface of cotyledon explant after torpedo stage.**Fig (2d)** Completely GUS expressing transformed somatic embryos **Fig (2e)** Transformed tomato plantlet maintained in selection media **Fig (2f)** Electrophoretogram of PCR amplification (nptII gene) from transformed plantlet leaf DNA lane one DNA ladder 1kb, Lane 2 positive control, lane 3 npt II 1.2kb amplicon from Leaf DNA sample as template

Fig.3

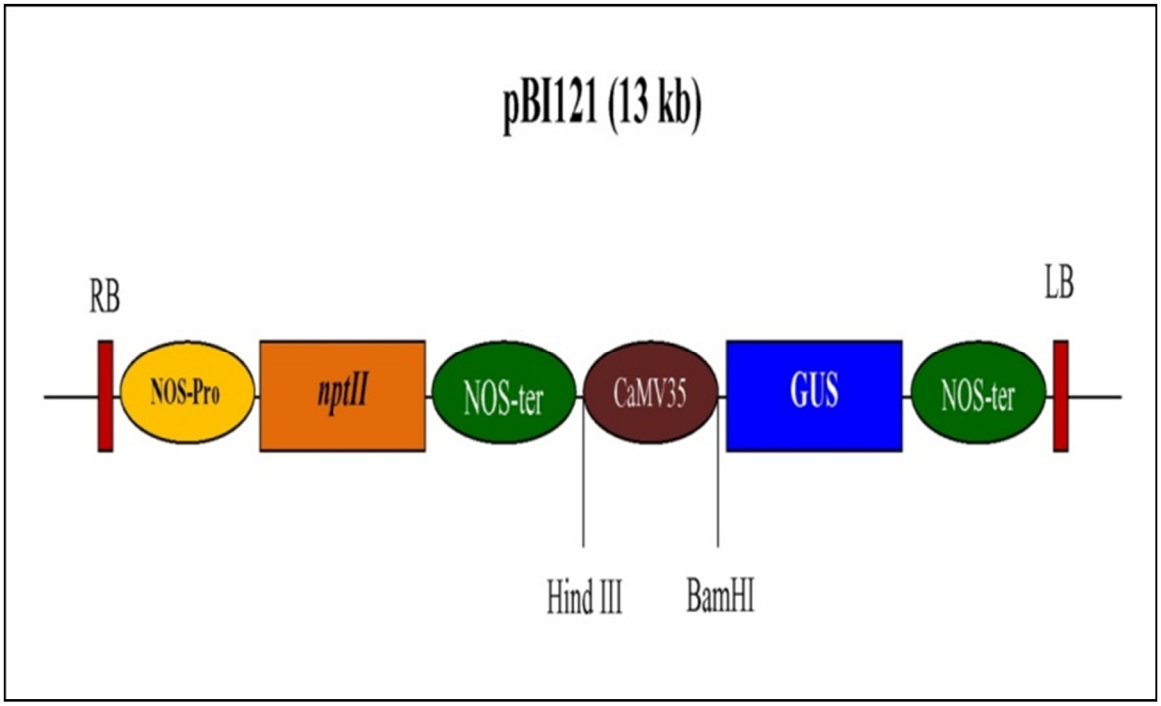

Fig (3)- Map showing the tDNA portion of pBI121 plant binary vector