



Construction of 6x myc tagged plant expression vector pBA 002 carrying *Rhizophora mucronata* Lam. specific glyoxalase I via homologous recombination

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Abstract: Plants are subjected to internal damage during stress conditions due to enhanced levels of methyl glyoxal (MG). Glyoxalase enzymes play the key role in MG detoxification and help the plant to survive. The glyoxalase system of *Rhizophora mucronata* Lam. was decoded; characterized and salt dependant increase in gene expression was analyzed in our previous studies (GenBank Accessions GGEC01061405, GGEC01044968, and GGEC01022591). In order to utilize these stress responsive genes in crop improvement, it is needed to monitor their methylglyoxal detoxification efficiency *in vivo*. For this, over expression of the glyoxalase enzyme(s) in a model/crop plant system can be done. Construction of a binary vector carrying coding region of glyoxalase gene(s) which can replicate both in *E coli* and *Agrobacterium tumefaciens* is the prime step in plant transformation research. In the present study *in silico* cloning of glyoxalase I, II and III specific to *R. mucronata* (Rm GLY I, Rm GLY II and Rm GLY III) were performed into pBA 002 plant expression vector carrying 6x myc insert. The binary vector is linearized with BsrG1 restriction enzyme. Cloning primers for all the three glyoxalase coding regions with 5' end terminal homology to the linear myc pBA were synthesized and validated *in vitro*. To account for *in silico* cloning, the Rm GLY I insert was successfully cloned via homologous recombination into myc pBA. The presence of Rm GLYI insert in the final construct was confirmed by colony PCR and sequence analysis.

Keywords: Mangrove; *Rhizophora mucronata*; Salt tolerance; Glyoxalase; Expression vector; pBA

Introduction

Molecular cloning is an inevitable recombinant DNA technique in modern molecular biology research. In routine cloning reactions, the restriction digestion of vector and gene inserts are performed followed by their fusion using DNA ligase. Polymerase chain reaction (PCR) facilitates the preparation of cloning gene inserts by selective amplification and addition of specific restriction sites. These gene fragments can be then inserted into any desired cloning vectors including those with expression capability in plants (Kelwick *et al.*, 2014). The plant expression vectors utilize *Agrobacterium* based phyto pathogenicity to modify plant genome and thus explore functional aspects of plant proteins.

These soil pathogens upon infection can transfer virulence genes of their genome into the plant cell. The afore mentioned peculiarity enables researchers to transform model/crop plants as per their assay design. The PCR amplified gene insert is incorporated first into a plasmid and then into an *Agrobacterium* carrying a second plasmid harboring virulence genes. This binary vector system is responsible for carrying, transferring and expressing the desired gene in the infected plant. It is difficult to culture and propagate *Agrobacteria in vitro*. Hence the initial sub cloning and the clone verification are usually performed in *E. coli* (Glick and Thompson 1993).

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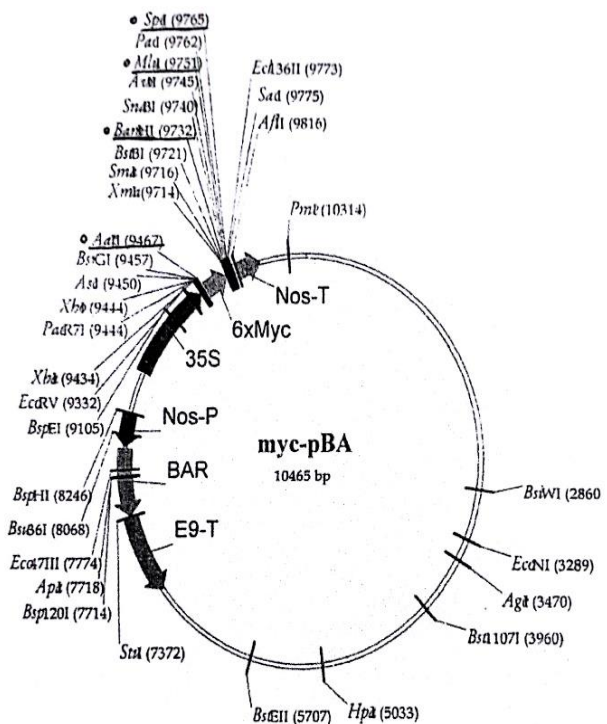
The cloning reactions involving restriction digestion and ligation are laborious involving too many steps often lead to unexpected results (Kelwick *et al.*, 2014). It is complicated to determine whether a particular restriction enzyme site is present or absent and to confirm the position of the site when present. To eliminate such difficulties, PCR based cloning strategies have been developed. The more advantageous among them is the homology based recombination reactions which utilize the sequence similarity at the end terminals of amplified gene insert and the linear vector. Once the fragments with required homology are ready, *in vitro* recombination is performed with the help of recombinase enzyme (Zhang *et al.*, 2012; Hartley *et al.*, 2000; Li and Elledge 2007; Geu-Flores *et al.*, 2007). Though it is evident that the efficiency of recombinational cloning is more efficient than conventional cloning, the former is least used by molecular biologists (Jacobus and Gross 2015). Routine restriction digestion and ligation cloning in myc pBA has been established already. In the present study recombinational cloning of mangrove glyoxalase gene(s) into a plant expression vector pBA 002 was carried out *in silico* as well as *in vitro*.

Materials and Methods

Vectors, control inserts

The binary vector used for cloning is myc pBA of length 10465 bp (Gifted by Dr. Sajeesh K, Department of molecular biotechnology Konkuk University, Seoul). It is a modified form of pBA 002 vector (Fig.1). The 6X myc tag is positioned in such a way that it is co transformed with the gene insert resulting in expression of myc tagged proteins in plants. The tag enables identification and purification of the insert gene product from the plant body (Hillman *et al.*, 2001). The gene for Spectinomycin (Antibiotic) resistance is the key for bacterial selection. The gene encoding phosphinothricin acetyl transferase, giving resistance to BASTA

(Herbicide) acts as a plant resistance marker (Block *et al.*, 1987).



Binary Vector : pBA002 x Asc I x Pac I
 Insert : 6xmyc x Asc I x Pac I
 Bacterial resistance marker : spectinomycin;
 Plant resistance marker : BASTA
 C125; E9 :: BAR :: Pnos - 35S :: MCS :: myc (9490-9705) :: MCS :: Tn

Figure 1. Vector map of pBA002 with myc gene insert

For cloning control reactions, a 7,266-bp linear conjugative cloning vector pYES7L and control lacZ insert were used. The pYES7L can replicate in a broad host range due to the presence of oriV and trfA gene. (Figurski and Helinski 1979) The control lacZ insert is 1.6 kb long carrying the lacZ gene and sharing a 15-bp homology with the linear pYES7L vector at both ends. The transformation efficiency of one shot DH10B T1 SA chemically competent *E coli* cells were assessed using pUC19 control DNA. The reaction serves as a transformation control. The cloning control vector and inserts were obtained from GeneArt Seamless plus Cloning and Assembly kit (Thermo scientific).

Plant material

Taxonomical identity of *Rhizophora mucronata* was confirmed by analyzing morphological features like leaves with elongated (mucronated) tips, axillary clusters of flowers on twigs, cream colour calyx with four sepals and four white, hairy petals and viviparous seeds. The leaf sample of *R. mucronata* with TaxId 61149 has been deposited under NCBI BioSample accession SAMN05846347. Leaves of *R. mucronata* were collected from Dharmadam estuary (Kannur, Kerala; Coordinates: 11°48'17.7"N 75°27'59.3"E). The leaves were processed immediately after sampling for RNA extraction.

Total RNA isolation, purification of and first strand cDNA synthesis

All glass wares and plastic wares used for RNA isolation were treated with 0.1% diethyl pyrocarbonate (DEPC) overnight and autoclaved for 2 hours. The work area and the micro tube racks were thoroughly wiped successively with Alcohol (70%) and RNase ZAP (Sigma) to make them RNase free. Water used for rinsing and reagent preparation was also made RNase free by adding 0.1% DEPC and incubating at least 16 hours before autoclaving. The RNA extraction and purification were performed following LiCl-cold extraction method (Rubinopina and Zapata-Perez 2011). Following RNA isolation, DNase treatment was done to remove genomic DNA contamination if any. The RNA preparation after DNase treatment was subjected to standard phenol/chloroform extraction. RNA was then precipitated by adding 0.1 volume of sodium acetate (3 M) and 2 volumes of ethanol (100%). Pellets were washed with ethanol (70%) and air dried. Dried pellets were finally dissolved in 20-50 µl of DEPC water. The purified RNA was quantified, purity analyzed (Bio-photometer, Eppendorf) and used as a template for reverse transcriptase enzyme for the synthesis of complementary DNA.

Plasmid isolation and purification of myc pBA vector

The *E coli* glycerol stock cultures carrying the vector myc pBA were revived in LB broth containing 100µg/ml spectinomycin (37°C, Overnight). Bacterial cells were harvested by centrifugation at 6800g for 2 minutes at room temperature. Plasmid purification was performed following Thermo Scientific GeneJET Plasmid Miniprep Kit user manual. All steps were carried out at room temperature. All centrifugations were done at 12000g.

Construction of linear myc pBA 002 vector

The vector sequence was linearized using single restriction digestion with Bsr GI. The recognition site of BsrGI restriction enzyme is T[^]GT-ACA. The digestion mix contained 16 µl nuclease-free water, 2 µl 10X tango buffer, 1 µl purified vector DNA (1 µg/µl) and 1 µl Bsr GI. All the components were mixed gently and spinned down for a few seconds. The final mix was incubated at 37°C for 4 hours. Inactivation of Bsr GI was achieved after the restriction digestion by incubating at 65°C for 20 min. To verify complete digestion and the size the restriction digestion products were analyzed using 1% agarose gel electrophoresis (Jordens, 1991).

In silico design and assembly of Rm GLY I, Rm GLY II and Rm GLY III coding region into myc pBA 002 using the strategy of homologous recombination

The primer designing and the coding fragment assembly were performed using GeneArt Primer and Construct Design Tool. The homology for recombination was generated by the PCR primers used for amplifying the glyoxalase encoding genes. PCR primers were designed so as to add 15 nucleotide overhangs on the 5' ends of gene inserts. The overhang provides the required end-terminal homology with the 3' end of linear vector sequence. The specificity of designed primers to the templates was analyzed

with the help of Primer BLAST. The primer sequences were then synthesised from Integrated DNA Technologies (IDT). Stock solutions of each primer pair were prepared at a concentration of 100 μ M nuclease free water. The glyoxalase gene fragments were individually assembled 5' to 3' into the linear vector sequence.

Preparation of cloning DNA inserts by PCR

The annealing temperatures of forward and reverse primers and the amplification conditions for Rm GLY I, Rm GLY II and Rm GLY III ORF were standardized. Phusion High-Fidelity DNA polymerase having 52-fold greater fidelity and 50-fold lower error rate than that of Taq DNA polymerase was used for amplification to ensure the quality of cloning. The PCR reactions were performed in 2720 thermal cycler, Applied biosystems. The prepared DNA inserts were verified by 1% Agarose gel electrophoresis.

***In vitro* cloning of Rm GLY I ORF into myc pBA 002 vector via homologous recombination**

The approval for cloning Rm GLY I insert into myc pBA plant expression vector system was granted by the Review Committee on Genetic Manipulation (RCGM; No. BT/BS/17/460/2012-PID). Cloning reactions were performed *in vitro* following GeneArt Seamless plus Cloning and Assembly kit user manual. The reaction mix (10 μ l) contained Rm GLY I gene insert (200 ng), linear myc pBA vector (50 ng), deionized water and enzyme mix. Control lac Z insert and pYESL vector instead of Rm GLY I and myc pBA respectively in the above mentioned reaction mix served as positive control. The positive control reaction mix without the enzyme mix was used as negative control. The cloning mix was incubated at room temperature for 60 minutes followed by incubation on ice for 5 minutes.

Transformation of chemically competent *E. coli* cells with the assembled construct

The transformation reactions were also performed following GeneArt Seamless plus Cloning and Assembly kit user guidelines. In brief, the seamless cloning and assembly reaction (3 μ l) was added to a vial of chemically competent *E. coli* (One shot DH10B T1 SA, Thermo scientific) and mixed gently by tapping the tube several times. In a separate vial of one shot DH10B T1 SA, 2.5 μ l of pUC19 Control DNA was added to perform transformation control reaction. The transformation mix was incubated first on ice for 30 minutes and then in a 37°C water bath for exactly 10 minutes. Re cooled the tubes on ice and incubated for 2 more minutes. 250 μ l of room temperature S.O.C medium was added to the transformation mix and kept in a horizontal incubator (200 rpm) at 37°C for 1 hour. From each transformation 150 μ l was added on pre-warmed LB plates containing 100 μ g/ml spectinomycin. The plates were incubated overnight at 37°C.

Analyzing transformants by colony PCR and sequencing

The positive transformants were directly analyzed using PCR with the help of Rm GLY I forward and reverse primers (Woodman *et al.*, 2016). The individual colonies used for colony PCR were picked with a sterile pipette tip and streaked on respective LB plates containing 100 μ g/ml spectinomycin for preparing glycerol stocks. The remaining cells in the pipette tip were diluted in 15 μ l nuclease free water by pipetting up and down. The cell suspension was boiled for 5min, followed by centrifugation at 13,000g for 10 min. The supernatant (2 μ l) was used as the template for colony PCR. The amplification conditions standardised for Rm GLY I insert preparation was used as such replacing template in a 25 μ l reaction.

The results were visualized by agarose gel electrophoresis (1%). The amplicon was sequenced by Sanger's method and then aligned using BLAST to the Rm GLY I sequence for confirmation (Altschul *et al.*, 1997; Sanger *et al.*, 1977)

Long-Term Storage of the Rm GLY I-myc pBA construct

As mentioned earlier each colony used for colony PCR was streaked on LB plates containing 100µg/ml spectinomycin to use for preparing glycerol stocks. Once the final Rm GLY I-myc pBA construct containing clone was finalized, it was selected for long term storage. A single colony was inoculated in LB broth containing 100µg/ml spectinomycin. The cells were grown until the culture reaches the stationary phase (OD₆₀₀ = 1-2). The culture (1 ml) was mixed with 50% sterile glycerol (1 ml) and stored as glycerol stocks at -80°C in cryovials (Howard 1956).

Result and Discussion

Preparation of glyoxalase gene inserts and linearization of myc pBA binary vector

The amplification conditions for Rm GLY I, Rm GLY II and Rm GLY III ORF with homology added primers were standardized using Phusion High-Fidelity DNA polymerase [98°C-2min, (98°C-10 s) 35 cycles, 53.1°C (Rm GLY I)/52.7°C (Rm GLY II)/51.4°C (Rm GLY III) - 30s, 72°C- 40s, 72°C- 10min]. The primer sequences

and template specific melting temperatures are given in Table 1. Discrete single bands were obtained at corresponding base pair position upon 1% Agarose gel electrophoresis (Fig. 2). The myc pBA vector plasmid was purified from overnight grown stock culture. The vector is then opened up at T[^]GTA -CA sites using Bsr G1 restriction enzyme. The size of linear vector was confirmed by comparing to 1 kb DNA ladder (Fig. 3).

In silico strategies of homologous recombination of Rm GLY I, Rm GLY II and Rm GLY III ORF into the myc pBA vector sequence

The required end-terminal homology for recombination is fixed as 15 bp as the hypothetical assembly consist of inserts <10 kb for a final construct of <13 kb. For bigger constructs an end-terminal homology >15bp is required for effective recombination. The final constructs carrying gene inserts in frame to the vector sequence were generated from the Gene art seamless plus cloning and assembly platform. This ensures the proper *in vitro* homologous recombination of Rm GLY I, II and III ORF into the myc pBA vector sequence. The Rm GLY I-myc pBA construct was found to be of size 11334 bp (Fig. 4A). The final construct size for Rm GLY II- myc pBA and Rm GLY III- myc pBA were 11448 bp and 11184 bp respectively (Fig. 4B and 4C).

Table 1. Cloning primers for Rm GLY I, Rm GLY II and Rm GLY III with end terminal homology to myc pBA

Oligo Name	Oligo Sequence	Oligo Size	Tm (Template Specific)
Rm GLY I_FW	GCTCGAGGGCGCGCCTATGGCAGAGGCTGTG	31	53.1
Rm GLY I_RV	TACCGTCGAGACGTCTTTACTGCAGCTCCATGA	33	53.1
Rm GLY II_FW	GCTCGAGGGCGCGCCTATGCAAATGATCTCAAAAGC	36	52.7
Rm GLY II_RV	TACCGTCGAGACGTCTTTAGAAGTTATCTTTTGCTCG	37	52.7
Rm GLY III_FW	GCTCGAGGGCGCGCCTATGACACATGTTTTATGGC	35	51.4
Rm GLY III_RV	TACCGTCGAGACGTCTCTACAGCTGTATGAACAGA	35	51.4

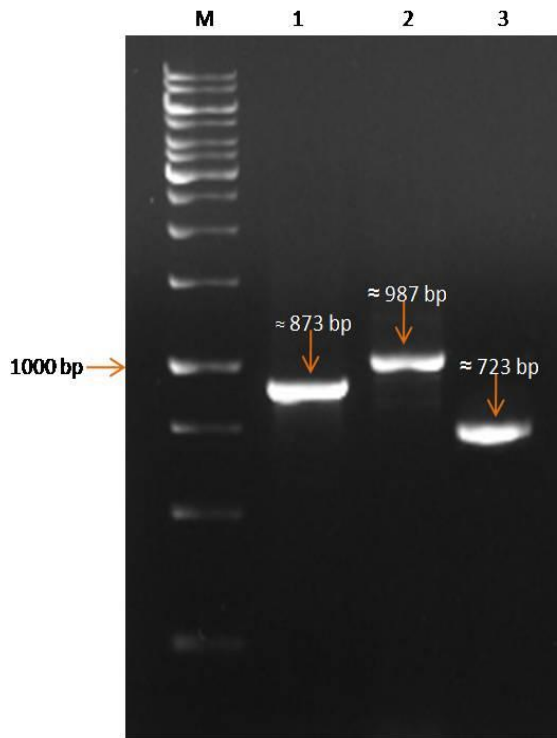


Figure 2. PCR amplified glyoxalase gene inserts in 1% Agarose gel. Lane M- 1 Kb DNA ladder, lane 1- Rm GLY I (873bp), 2- Rm GLY II (987bp) and 3- Rm GLY III (723bp)

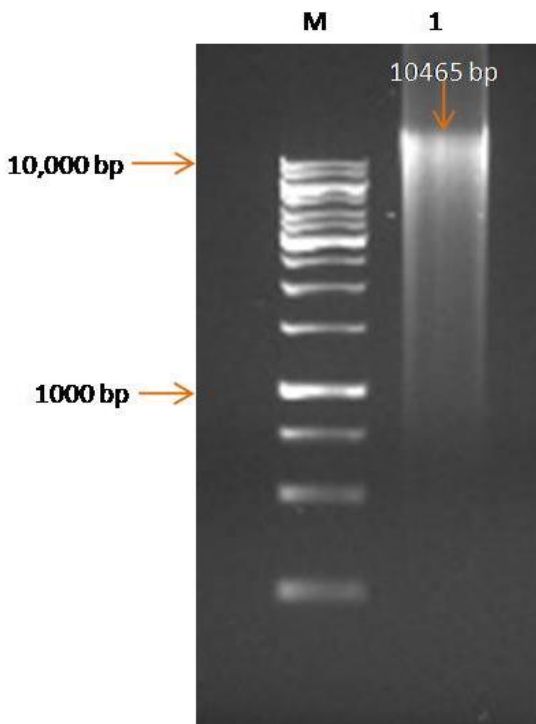


Figure 3. Bsr G1 digest in 1% Agarose gel. Lane M- 1 Kb DNA ladder, lane 2- Linear myc pBA vector (10465 bp)

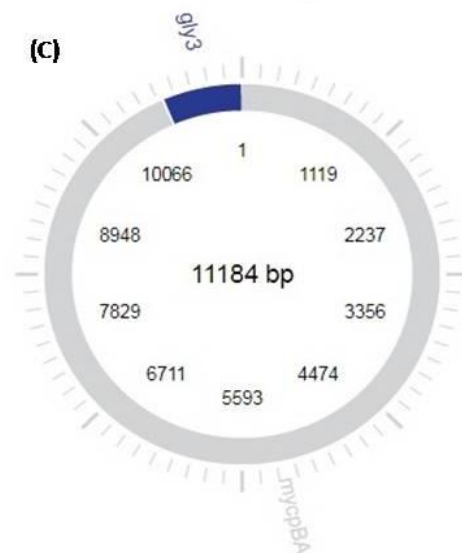
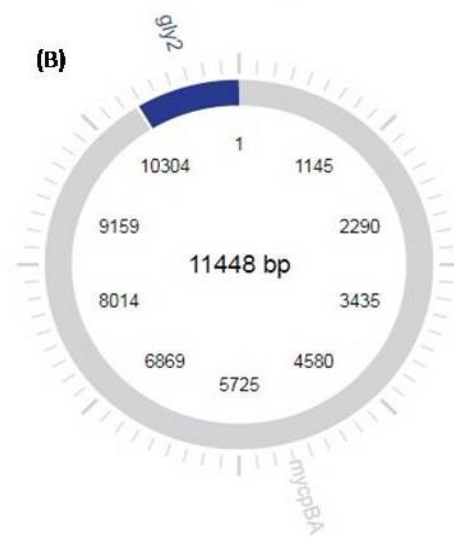
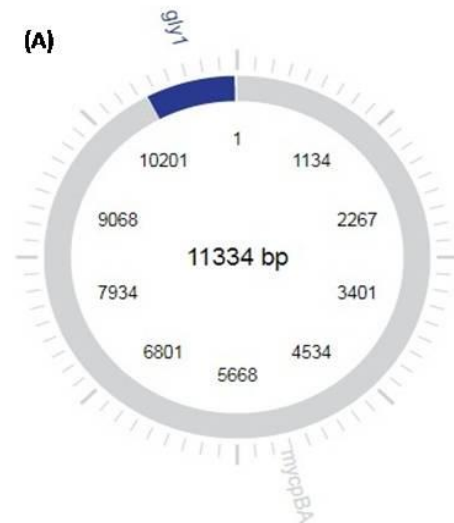


Figure 4. *In silico* cloning of (A) Rm Gly I, (B) Rm Gly II and (C) Rm Gly III ORF into myc pBA vector. The final construct size for (A), (B) and (C) are 11334, 11448 and 11184 respectively.

In vitro cloning of Rm GLY I and analysis of transformants

Genome information of most of the plants are not available till date. Hence for cloning and charactering a particular gene of interest, transcriptome and mRNA-based approaches are commonly utilized. In this study, glyoxalase I gene, specific for the mangrove species *R. Mucronata* was successfully cloned into linear myc pBA plant expression vector via homologous recombination. Compared to restriction ligation cloning, homologous recombination ensures exact orientation of insert in the final construct. For the expression of final construct in plant, it should be flanked by a promoter and terminator sequence. The open reading frame of Rm GLY I is of length 873bp which encodes 290aa protein. The Rm GLY I ORF excluding stop codon was inserted in between 35S CaMV promoter and 6X myc linked Nos terminator. The use of Rm GLY I has not been reported in plants for functional analysis/stress tolerance. The cloning of the amplified Rm GLY I gene insert and the subsequent transformation yielded recombinant white colonies (Fig. 5D). Colony PCR using insert specific primers gave amplicon at the desired position (Fig. 6) which on sequencing gave the exact Rm GLY I gene sequence.

Positive and negative control reactions were performed in parallel to assess the reaction fidelity. Control lacZ Insert was cloned into the linear pYES7L vector to yield numerous white colonies in the positive control and blue colonies in the negative control (Fig. 5A and 5B). The transformation control also gave blue colonies as expected (Fig. 5C). Compared to the control reactions, the transformation efficiency of Rm GLY I- myc pBA construct was low which might be due to >10 Kb construct size. Glycerol stocks are maintained for long term storage of Rm GLY I- myc pBA clones. The protocol standardized for Rm GLY I cloning

can be followed as such for other two glyoxalase enzymes of *R. mucronata*.

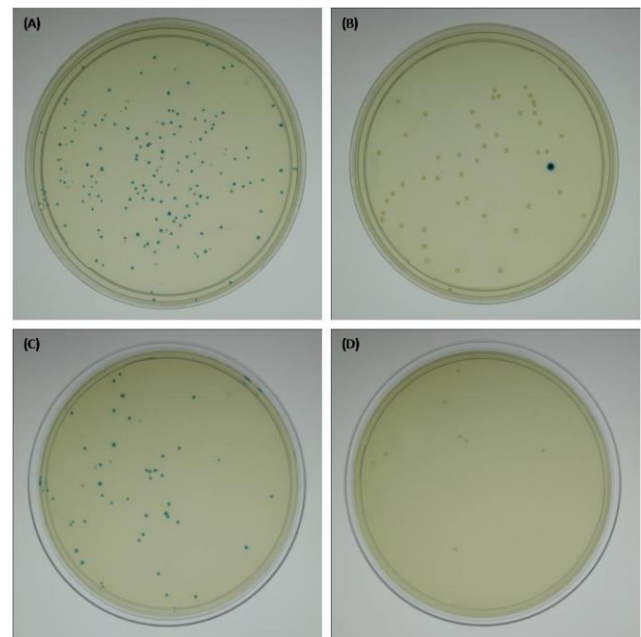


Figure 5. Blue white screening of transformed one shot DH10B T1 SA chemically competent *E. coli* colonies on LB agar plate (A) Positive cloning control (B) Negative cloning control (C) Transformation control (D) Rm GLY I myc pBA transformants

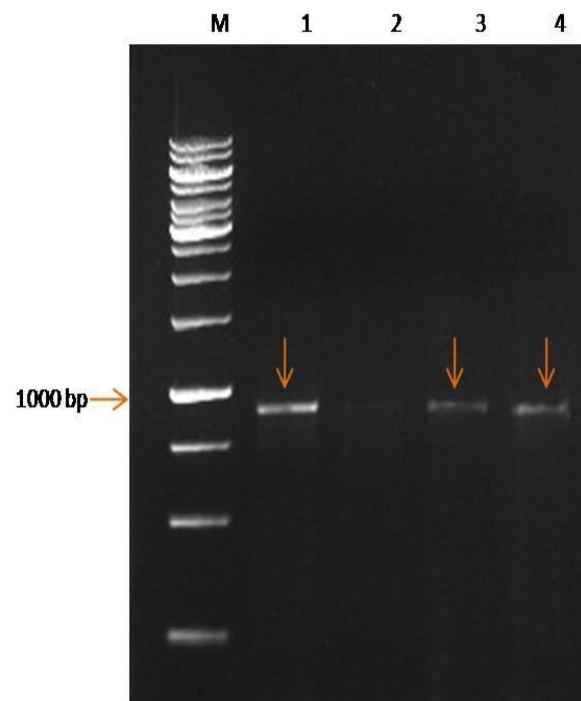


Figure 6. Colony PCR showing Rm GLY I gene amplicon of size 873bp.

The Rm GLY I-myc pBA construct prepared may be directly used for transient or stable transformation of model or crop plants through *Agrobacterium* mediated techniques. These results also strengthen the idea that homologous recombination in *E. coli* might be one of the most effective methods for cloning PCR fragments.

Conclusion

The cloning strategies for Rm GLY I, Rm GLY II and Rm GLY III coding region into a myc tagged plant expression vector (myc pBA 002) via homologous recombination is proposed in this study. Plasmid construct of myc pBA 002 carrying Rm GLY I is also prepared making it ready for use to transform any crop plant for glyoxalase induced salinity tolerance.


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