

Modification of Recombination-based GATEWAY™ Binary Destination Vector with Novel Promoter for *Agrobacterium*-mediated Transformation of Rice

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Abstract

The GATEWAY™ Binary Destination Vector pH7WG2 is available for easy insertion of genes for transformation into plants. The gene of interest integrates downstream of the Cauliflower Mosaic Virus Promoter CaMV 35S by recombination. The CaMV 35S promoter is however not suitable for transformation and expression of genes in monocots like rice. We isolated and cloned a ~1100 bp upstream region from two rice (Pokkali and IR64) Na/H antiporter genes into the GATEWAY™ promoter cloning vector pHGWFS7. The Pokkali promoter expressed the β -glucuronidase or GUS gene ~25-fold more efficiently than the CaMV 35S promoter in rice calli, while that of IR64 was 7-fold more. The IR64 promoter however showed efficient expression in transgenic rice leaves. The promoter from Pokkali Na/H antiporter was used to replace the CaMV 35S sequence in pH7WG2. The CaMV 35S region was cut out and the linear vector fragment blunted and T-tailed. After amplification of the promoter from Pokkali rice DNA, it was A-tailed and ligated to the modified T-vector. The resultant vector, named pH7WG3, following the nomenclature at the gateway site, www.plantgenetics.rug.ac.be/gatewayT, can now be used for recombination of any genes for efficient rice transformation.

Introduction

GATEWAY™ Technology uses bacteriophage lambda-based site-specific recombination (Hartley et al. 2000) instead of restriction endonuclease and ligase to insert a gene of interest into an expression vector. The advantage of this system for cloning is profound since it is common to find that suitable restriction enzyme sites, even if created using PCR, will often cut the gene of interest. The DNA recombination sequences (*attL*, *attR*, *attB*, and *attP*) and the Clonase

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enzyme mixtures, LR or BP Clonase (Invitrogen) mediate the lambda recombination reactions. Four types of sites are involved in two reactions as follows: the BP reaction, $attB \times attP \rightarrow attL + attR$ and the LR reaction, $attL \times attR \rightarrow attB + attP$. All *att* sites contain a central 7-bp overlap region, defined by the integrase cleavage site that largely dictates the specificity of the recombination reaction and up to 4 variations of the BP or LR *att* sites are known (Landy 1989, Cheo et al. 2004). Reactions in which unique *att* sites flanking a gene of interest (GOI) such as *attB1*-GOI-*attB2*, are reacted with a vector that includes compatible sites (*attP1*-*attP2* sites) will result in sequential recombination reactions that transfer the GOI into the selected vector backbone, now flanked by *attL* sites (*attL1*-GOI-*attL2*). Because the recombination events that occur within the 7-bp overlap regions are precise, the reading frame and orientation of the transferred DNA are maintained, providing for amino- and/or carboxy-terminal fusion proteins to be rapidly constructed, and conserving the integrity of the transferred DNA sequence. The utility of the system has been enhanced further by introduction of entry and destination vectors, which will allow simultaneous insertion of two, three or four fragments in 1-2 reactions (Karimi et al. 2005).

PCR products can also be directly inserted into TOPO or directional TOPO ENTR vectors (Invitrogen) by simple incubation reactions using topoisomerase flanked by *attL1* and *attL2* sites. The inserted DNA can then be mobilized into *Agrobacterium*-compatible binary expression-, promoter- or RNAi-based destination vectors containing *attR1* and *attR2* sites by LR recombinase for plant transformation. A number of these destination clones using both kanamycin and hygromycin as plant selectable markers are available at nominal charge (www.plantgenetics.rug.ac.be/gateway). The expression vectors available allow any GOI to be recombined downstream of the CaMV 35S promoter, which is adequate for transformation of genes into dicots. Expression of GOI using this promoter in monocots, such as rice, is however poor (McElroy et al. 1995). This work was undertaken to identify a promoter showing efficient expression in rice and replace the CaMV 35S promoter in the Gateway destination expression vector, pH7GW2, with the rice promoter.

Materials and Methods

A versatile set of vectors are available for plant gene transformation and analysis (Karimi et al. 2002). For LR recombination reactions, Entry vectors contain the site specific sequence *attL1*-*attL4*, while the destination vectors contain *attR1*-*attR4*. Genes or promoters of interest are cloned into the Entry vector by ligase-free topoisomerase reactions, (Invitrogen Topo-cloning). Thereafter, recombination between the two vectors using LR recombinase allows recombination of the

gene of interest or promoter into the appropriate location on the destination vector.

Destination vectors: Two types of commercially available destination vectors were used for transformation in this work.

(a) *pHGWF57 for promoter-gus constructs (Fig. 1)* : This vector with binary pPZP200 backbone (Hajdukiewicz et al. 1994) contains CmR-ccdB selection flanked by *attR1* and *attR2* recombination sites and followed by *gfp-gus* sequence with t35s termination sequence. The vector can be grown in the presence of chloramphenicol (Cm). To ensure that only recombinants grow, the cytotoxic *ccdB* gene gets replaced after recombination as well as the chloramphenicol resistance gene (CmR). Recombinants can also be selected by growth on streptomycin and/or spectinomycin. The recombination sites allow transfer of a promoter that drives the *gfp-gus* gene hence allowing its characterization and testing.

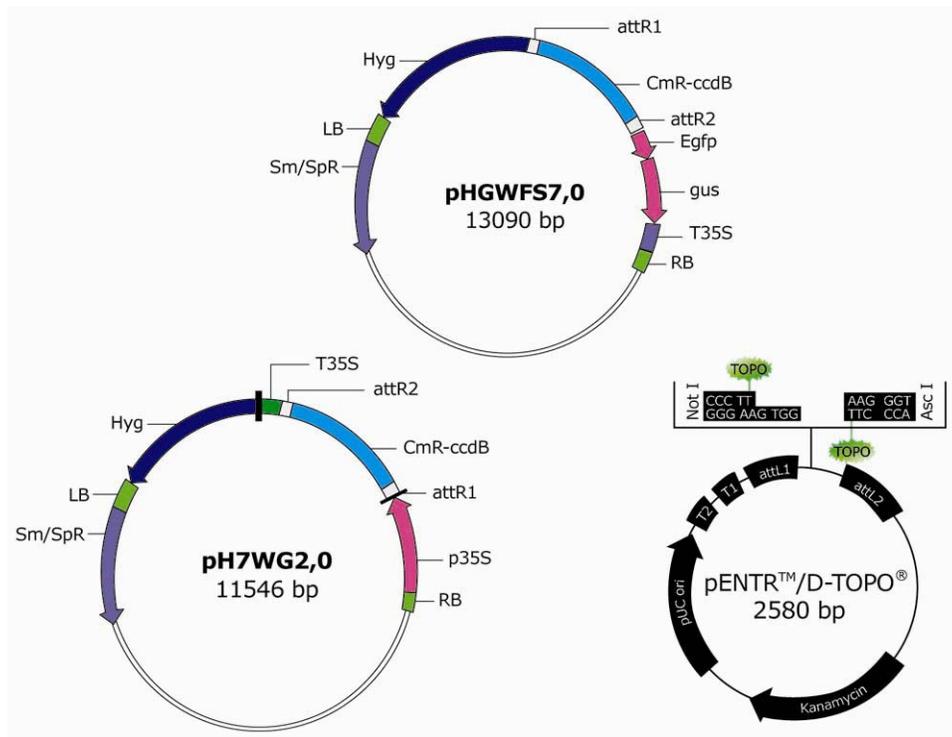


Fig. 1. Vectors pHGWFS7, pH7GW2 and pENTR/D, respectively.

(b) *pH7WG2 for gene transfer (Fig. 1)*: This vector has the same pPZP200 backbone but contains CaMV35S promoter followed by CmR-ccdB selection

flanked by *attR1* and *attR2* recombination sites. The recombinations sites allow transfer the gene of interest downstream of the CaMV 35S promoter.

Entry vector: The pENTR/D-TOPO entry vector (Invitrogen) was used for cloning of the promoter. The promoter was first amplified by PCR reaction using a CACC sequence upstream of the forward primer. This allowed strictly directional cloning of that promoter sequence. The attached topoisomerase at the end of the entry vector directs the ligation reaction without the help of ligase enzyme. The entry site of pENTR/D-TOPO is flanked by *attL1* and *attL2* sequence and therefore allows transfer of the promoter to the destination vector through recombination reaction.

PCR amplification of promoter: To amplify the promoter, genomic DNA from Pokkali and IR-64 variety were used as template. Amplification was carried out using PCR mix containing PCR reaction buffer (Invitrogen), 1.33 mM MgCl₂, 0.1 mM dNTP mix, 2.66% DMSO, 0.5U Taq DNA polymerase (Invitrogen), 0.05U Platinum *Pfx* and 50 ng each of forward (5'-CACCACACATGGAGCTGTCTCC-3') and reverse (5'-GTAGTCGGAGGTCGTAC-3') primer. This amplification mixture was used to amplify the promoter for cloning as well as confirming the transformed colonies. The PCR reaction was preceded with 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, with an extension of 72°C for 7 min. The amplification products were checked by 0.8% agarose gel electrophoresis. To check the orientation, another forward primer (5'-TTGAGCTCTCCCATATGGTCG-3') was designed from the upstream region of the vector homologous to the site of insertion and the above reverse primer.

Blunting reaction: After restriction digestion, (for CaMV 35S replacement) blunting (end filling reaction) of the vector was done with T4 polymerase (Invitrogen) using 1× T4 polymerase buffer, 0.5 mM dNTP mix and 5U T4 DNA polymerase for 5 µg of DNA in 20 µl reaction. The reaction mixture was incubated at 37°C for 1 hr. The reaction was terminated by phenol:chloroform (1:1) extraction and ethanol precipitation (3M sodium acetate, ice cold ethanol).

Tailing of DNA: T-tailed vector and A-tailed insert was generated using Taq polymerase (Invitrogen), 1X PCR reaction buffer, 1.33 mM MgCl₂ and 0.66 mM dATP and dTTP respectively. This mixture was incubated at 72°C for 10 minutes followed by phenol:chloroform (1 : 1) extraction and ethanol precipitation.

Transformation of E. coli (Heat shock): DNA to be transformed was mixed with the *E.coli* chemical competent cell and incubated on ice for 30 minutes. The mixture was heat-shocked for 90 seconds at 42°C without shaking and immediately chilled on ice. The cells were mixed with SOC media (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose) without antibiotic and incubated at 37°C with shaking (200 rpm)

for 1 hr. The mixture was then spreaded on appropriate antibiotic containing LB agar plate.

Transformation of plants: Agrobacterium tumefaciens (strain LBA4404) containing the promoter-GUS construct (pHGWS7) was used to transform rice calli. The transformation method was essentially carried out according to the method described by Khanna and Raina (1999) with some modifications/optimization described below (Rasul et al. 2005).

Agrobacterium culture and preinduction: Agrobacteria containing promoter-GUS constructs were streaked out on AB medium (Chilton et al. 1974) supplemented with 50 mg/l kanamycin (for pCAMBIA-1305.1) or 20 mg/l streptomycin and 50 mg/l spectinomycin (for pHGWS7) and incubated at 28°C for 48-72 hours, and a single colony was grown with 250 µM Acetosyringone. Bacteria were collected by centrifugation. The supernatant was removed and the bacteria washed in 10 ml of Bacterial Resuspension Medium (AB medium with 36 g/l glucose, 68.5 g/l sucrose, AS 250 µM, 50 mg/l kanamycin, pH 5.2), pelleted and resuspended again in 10 ml of bacterial resuspension medium. The Optical Density (550 nm) was adjusted to 1.0 for Binnatoa and 0.4-0.7 for BRRI dhan 34.

Callus induction, subculturing and differentiation: Dehusked and sodium hypochlorite sterilized seeds were plated on MS induction medium according to Seraj et al. (1997) at 25 - 27°C in the dark for 21 - 30 days. Pieces of actively proliferating scutellar calli (2-3 mm diameter) were isolated and subcultured on fresh MS induction medium for 3 days prior to preincubation. Calli were preincubated for 72 hours in semisolid Explant Preincubation Media [MS induction medium except 2, 4-D plus 0.5 mg/l BAP, 0.5 mg/l NAA, 12.5 mM phosphate buffer (K₂HPO₄-NaH₂PO₄; pH 5.6)]. Calli were transferred back to fresh MS induction medium for 2-3 days before infection, immersed in bacterial suspension (OD. 0.4-0.7) for 10 minutes and excess removed before transfer to semisolid co-cultivation media (Explant preincubation medium, Acetosyringone 500 µM, 3 g/l Phytigel, pH 5.8). After incubation at 25°C for three days in the dark the calli were assayed for transient GUS activity or transferred to callus selection media [MS induction medium, 50 - 100 mg/l hygromycin, different concentrations of 300 - 500 mg/l Carbenicillin, 200 - 300 mg/l Cefotaxime]. Regeneration of plants was carried out on a differentiation medium (Seraj et al. 1997).

Assays of transgenic calli and plants: The transgenic status of calli was detected by both qualitative and quantitative GUS assays and of plants by PCR analysis and qualitative GUS assay.

PCR analysis: DNA was extracted from transformed rice leaves using modified CTAB method (Doyle and Doyle 1990) and PCR was done to detect the integration of the hygromycin phosphotransferase (*hpt*) gene with the primer

pair Long_HPT_F (5'-CGAAGAATCTCGTGCTTTCAGC-3') and Long_HPT_R (5'-AGCATATACGCCCCGAGTCG-3'), present in the T-DNA region of pHGWS7. A 25 μ l reaction mixture containing 100 ng of plant DNA, 100 μ M of each dNTP, 2.4 ng each of primer: Long_HPT_F and Long_HPT_R, 1 unit of *Taq* DNA polymerase (*Invitrogen*), 1.5 mM MgCl₂, DMSO 2.4% and 1X PCR Buffer-MgCl₂ (*Invitrogen*) was prepared for PCR assay. The PCR reaction was preceded with 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 65°C for 1 min, 72°C for 1 min, with an extension of 72°C for 7 min. The amplification products were checked by 0.8% agarose gel electrophoresis.

GUS activity assay: GUS activity in the rice calli was measured fluorometrically after 200 mM salt stress at different time points using 1 mM 4-methyl-umbelliferyl- β -D-glucuronide (MUG) as substrate (Jefferson et al. 1987), 1mM 4-methyl-umbelliferone (MU) as Assay Solution and 0.2 M Na₂CO₃ (Stop Buffer) by machine TURNER 450 Fluorometer. GUS Extraction Buffer (50mM NaPO₄ of pH7.0, 10mM beta-ME, 10mM Na₂EDTA of pH 8.0, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100, 25mg/L PMSF (~0.14mM)) was used to extract GUS from plant samples. In our experiment, uninfected calli were used as the sample blank. From the standard curve of fluorescence vs. concentration of MU, the MU formed by unknown GUS sample was identified.

To find out specific activity of GUS protein (unit: μ moles MU mg/min), total protein was estimated by Lowry Method (Lowry et al. 1951) as well as Hartree-Lowry Assay (Hartree 1972). Each three replicates from the samples under same condition were averaged and standard deviations (STDDV) for the samples were determined using Microsoft Excel program.

GUS activity in the Control and transformed calli with endogenous promoters was also measured histochemically (Jefferson 1987) using the indigogenic substrate 1 mg/ml X-gluc (5-bromo, 4-chloro, 3-indolyl β -D-glucuronide). Calli were fixed at pH 5.6 by vacuum infiltration for 2 minutes submerging them into 10 mM MES, 0.3% formaldehyde and 0.3 M mannitol. The tissues were then washed several times by 50 mM phosphate buffer and incubated in X-gluc for 16-72 hours at 37°C and then decolorized using 99% ethanol. Buffers and substrate were filter-sterilized and sterile microcentrifuge tubes used for assay to avoid false positives from *E.coli* contamination. In our experiment, uninfected calli were used as negative control and transformed tobacco leaf was used as positive control.

Results and Discussion

Isolation of promoter: Commercially available destination vectors such as pH7WG2 allow the transfer of GOI downstream of the CaMV 35S promoter. Transformation with constructs in which the GOI is driven by the CaMV 35S

promoter, gives adequate expression in dicots like tomato and *Brassica* (Zhang et al. 2001, Zhang and Blumwald 2001). But in monocots like the rice the expression is very poor (McElroy et al. 1995, Fukuda et al. 2004).

In order to identify a good promoter for rice transformation, we have isolated the 1 kb upstream region of the Vacuolar Sodium Proton Antiporter gene (*OsNHX1*) from Pokkali and IR64 variety of rice (PkN and IRN respectively). The PCR primers for the amplification of these promoters were designed by comparing the 2000 bp upstream sequences of Pokkali, IR64 and Nipponbare variety. Conserved region at ~1000 bp upstream from the ATG codon among these three sequences was selected as primer. The PCR-amplified product from Pokkali (1109 bp) and IR64 (1090 bp) were cloned into the directional pENTR/D-TOPO vector and transformed into *E. coli* DH5 α , according to the kit manufacturer's instructions. Positive colonies were confirmed by PCR with the primer set that were used for amplification of the promoter (data not shown). Positive inserts were sequenced and submitted to GeneBank (Accession no. EF650026 for PkN and EF650027 for IRN). Transcription factor motif analysis revealed the presence of some extra enhancer elements in PkN and IRN (20 and 19 elements respectively) compared to Nipponbare (10 elements). MYB and calcium response elements (CRE) were found in both PkN and IRN whereas MYC found only in PkN, but all these motifs were absent in Nipponbare.

The two pENTR/D-TOPO vectors were individually incubated with pHGWFS7 vector to recombine the promoters at the upstream of GUS gene. Here, LR clonase was used as per provider instructions (Invitrogen) and the reaction products transformed into *E. coli* DH5 α . Clones were confirmed using primers homologous to the recombined sequence of *attP* (data not shown). PkN-GUS and IRN-GUS constructs thus produced were transformed into *Agrobacterium* and then into plants.

The integration of the hygromycin phosphotransferase (*hpt*) gene, present in the T-DNA region of pCAMBIA 1305.1 and pHGWFS7, in the transformed plants was detected with the Long_HPT primer pair. Fig. 2 shows amplification of the correct band (708 bp) in three transformed plants.

Characterization of promoters: Rice calli transformed with pCAMBIA 1305.1 (CaMV-GUS construct) and pHGWFS7 (PkN-GUS and IRN-GUS constructs) were stained with the histochemical dye, X-gluc (Fig. 3). The intensity of blue color was much greater in both the pHGWFS7 constructs, indicating strong gene expression capacity of the inserted promoters (PkN and IRN). Fluorometric data (Fig. 5) shows that PkN and IRN are at least 25 and 7 times more efficient than CaMV 35S respectively. The PkN promoter may have further inducibility under salt stress. However, this will need to be vigorously tested in intact transgenic

plants. Large variability was found in flourometric GUS activity in independent transformation events, although the difference in activity between CaMV 35S and PkN/IRN-driven expression was always maintained. We therefore, proceeded to use the pHGWFS7 for transformation and regeneration of rice plants. Differences in vector background between CaMV 35S-GUS (pCAMBIA 1305.1) and PkN/IRN-GUS (pHGWFS7) may also be responsible for part of the difference in GUS activity. Rice leaves transformed with IRN-GUS showed uniformly high GUS expression. PkN-GUS plants were found more difficult to regenerate. The ratio of the regenerated IRN-GUS and PkN-GUS plants was found to be 10 : 1. In the IRN rice roots, however, GUS expression was only observed in root hairs and region immediately above the meristematic zone (zone of elongation) (Fig. 4).

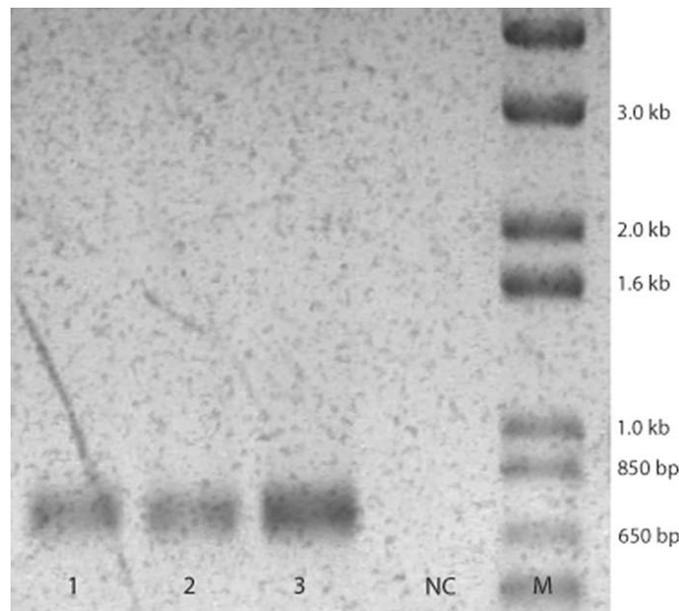


Fig. 2. Confirmation of transgenic status of plants by Long_HPT primer set producing 708 bp PCR amplicon. Lane 1 - 3: Transgenic plants, NC: Negative control, M: 1 kb plus ladder (Invitrogen).

Modification of destination vector: PkN gave extremely good GUS expression in rice calli and therefore was thought to be ideal for driving expression of GOIs in rice. We therefore, decided to replace the CaMV 35S promoter in the binary destination vector, pH7WG2, which is used for overexpression of genes preferably in dicot plants.

The construction involved cutting out of the CaMV 35S promoter using *Spe* I and *Sst* I (Invitrogen) from the commercial Gateway destination vector pH7WG2.

The digested fragment was separated by electrophoresis at 0.8% agarose gel and band was extracted using QIAGEN gel extraction kit as per manufacturer's instruction (Fig. 6). Extracted DNA containing an overhang generated by the restriction enzymes were blunted using T4 polymerase. An extra T nucleotide (T-tailing) was added to the blunted vector ends. Simultaneously, PkN was amplified by PCR reaction. Generally a PCR reaction adds an extra 'A' nucleotide. But in this case we used proofreading polymerase (Platinum *Pfx*, Invitrogen) along with *Taq* DNA polymerase (Invitrogen) in 1 : 10 (U/U) ratio

COLOUR

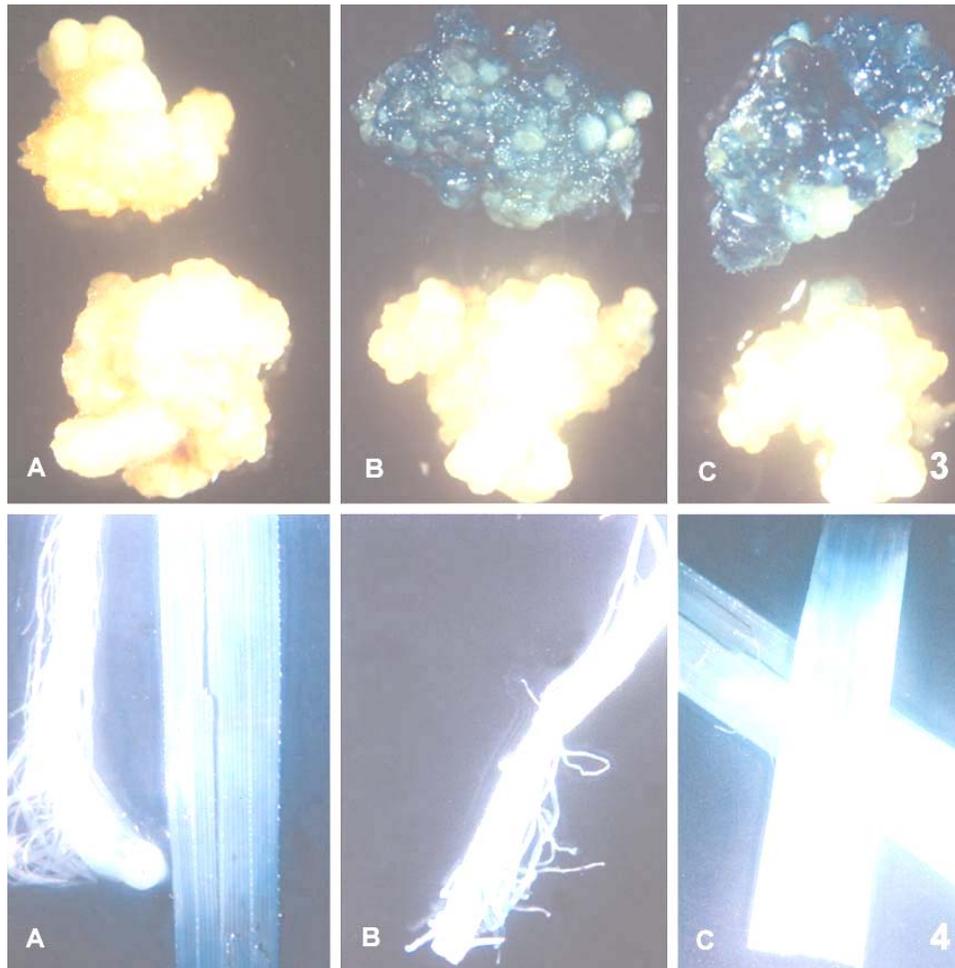


Fig. 3. GUS activity assay in rice callus driven by CaMV (A), IRN (B) and PkN (C) promoter. Fig. 4. Histochemical GUS assay in different tissues. (A) Root showing color at zone of elongation and outer face of leaf. (B) Color in root hair and (C) Inner face of leaf.

for PCR amplification which removes the extra A. Therefore, PCR product was precipitated and then A-tailed after redissolving. The T-tailed vector fragment and A-tailed PkN insert was mixed and incubated with T4 DNA ligase overnight (i.e. 16 hrs) at 16°C. This mixture was then transformed into *E. coli* by heat shock method. Successful transformants were grown on LB-agar plate containing Spectinomycin antibiotic. Successful cloning was also confirmed by PCR with the same primer set which was used for the isolation of promoter. Fig 7 shows band at 1kb from 4 colonies.

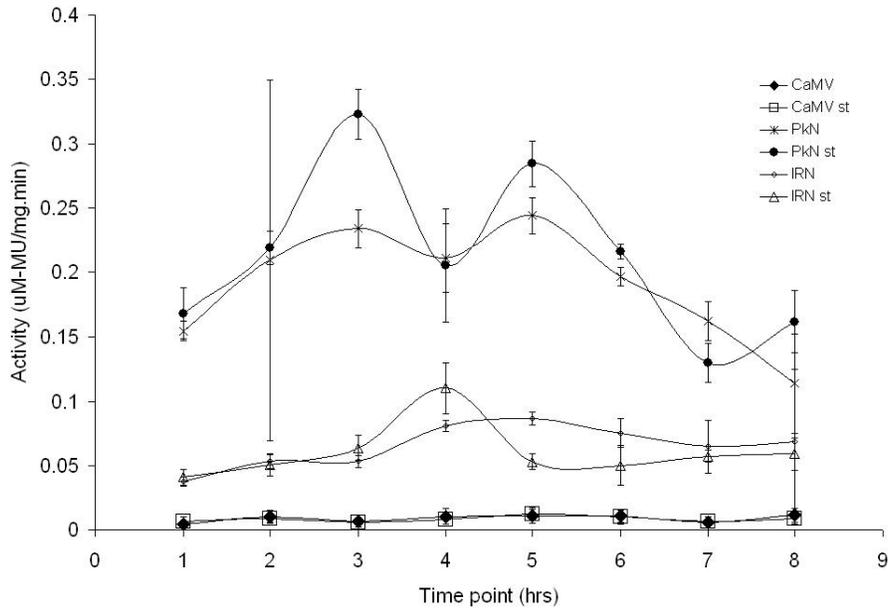


Fig. 5. Comparative fluorometric GUS activity assay of CaMV, PkN and IRN promoter at different time point under normal and 200 mM salt stress.

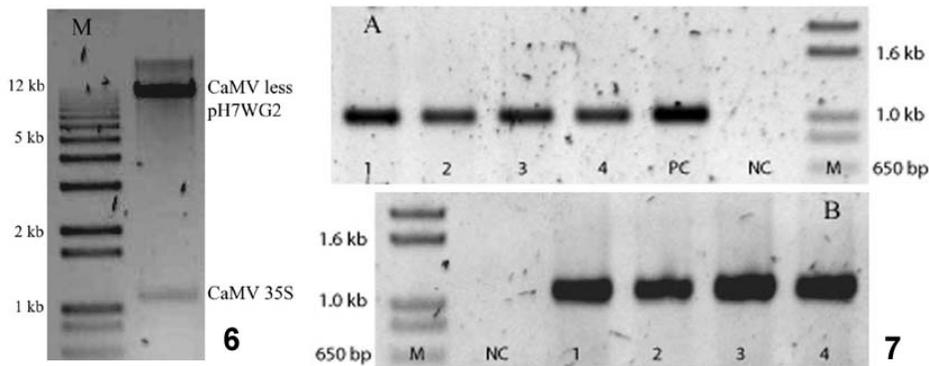


Fig. 6. Digestion of pH7WG2 destination vector with *Spe* I and *Sst* I to cut out CaMV 35S promoter. Fig. 7. Confirmation of transformation (A) and orientation of ligation (B). Gel

A: Lane 1-4 indicates PCR from plasmid of colony 1-4, PC: positive control and NC: water control. M indicates 1Kb plus molecular size marker (Invitrogen). *Gel B*: Lane 1-4 indicates PCR from plasmid of colony 1-4. NC: negative control. M indicates 1Kb plus molecular size marker (Invitrogen).

The TA ligation is not directional and so the insert may ligate in a flipped orientation which will result no activity of the promoter. To pick the ligation product with the correct orientation, a new forward primer was designed from the vector sequence, where the reverse primer was the same as for the amplicon. Only the clones with correct orientation gave PCR amplification (Fig. 7).

The new vector was named pH7WG3 (Fig. 8) and we will be testing its efficacy for gene expression by recombining GOIs downstream of the promoter PkN. This vector should prove to be useful for expression of GOIs in monocots like rice. Substituting CaMV 35S by PkN was difficult by restriction digestion-ligation method because the restriction enzyme used for cutting out of the CaMV 35S sequence digested PkN. Therefore we decided to blunt the restriction enzyme cut ends. However blunt end ligation is not efficient, therefore the vector was T-tailed and the insert was A-tailed. The process described in this work is therefore useful in transferring a DNA sequence into any site of interest.

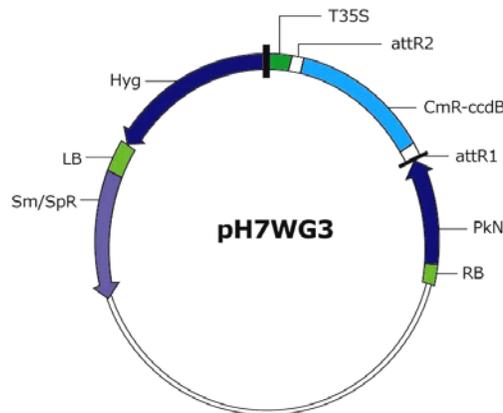


Fig. 8. Modified binary destination vector pH7WG3. CaMV 35S promoter of pH7WG2 is replaced by PkN.

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