

## Progeny analysis of transgenic rice variety transformed with *Glyoxalase I* gene

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### Abstract

Salinity is one of the major yields limiting factor that limit the worldwide productivity and distribution of cereal crops and the development of genetically engineered plants with enhanced tolerance to salt presents an important tool to increase the productivity. We introduced *glyI* (*glyoxalase I*) gene from *Brassica Juncea* into rice variety PR116 through particle bombardment for salt tolerance. Integration and expression analysis in T<sub>0</sub> and T<sub>1</sub> generations were confirmed through Polymerase chain reaction and *in vitro* screening for salt tolerance. In this study we confirmed the integration and expression of gene (*glyI*) in next two generation (T<sub>2</sub>, T<sub>3</sub>) through Polymerase Chain Reaction and methylglyoxal experiment. Expression of *glyoxalase I* gene was induced by different concentrations of methylglyoxal treatment and most of the transgenic lines were highly tolerant to methylglyoxal and showed high chlorophyll content after 72 h of treatment.

### Highlights

We introduced *glyI* (*glyoxalase I*) gene from *Brassica Juncea* into rice variety PR116 through particle bombardment and studied the integration and expression of gene (*gly I*) in progenies through polymerase chain reaction and methylglyoxal sensitivity experiment.

**Keywords:** Salt stress, glyoxalase I gene, transgenic rice, polymerase chain reaction.

More than a third of all the irrigated agriculture land in the whole world is affected by salt stress and loss from the salt stress is effects the food supply of world population. Abiotic or salt stress tolerance could be improved by conventional breeding techniques or through introducing stress resistance genes for salt tolerance. Genetic manipulation of crop plants for enhanced abiotic stress tolerance holds great promise for sustainable agriculture (Altman, 1999). They have been shown to have a quantitative character and thus they are controlled by multiple genes (Hasegawa *et al.*, 2000; Atkinson *et al.*, 2013). Present engineering strategies for salinity tolerance rely on the transfer of one or more genes that are either involved in the

signaling pathways or that encode enzymes required for the functional and structural protectants such as osmolytes and antioxidants or that encode the protein that confer stress tolerance (Garg *et al.*, 2002; vinocur *et al.*, 2005; Jewell *et al.*, 2010).

The glyoxalase system consists of two enzymes *glyoxalase I* and *glyoxalase II*. These enzymes act coordinately in a two step reaction to convert cytotoxic methylglyoxal and other 2-oxoaldehydes to their 2-hydroxyacids using GSH as a cofactor (Kwon *et al.*, 2013). Glyoxalase I first catalyses the formation of S-D lactoglutathione from hemithioacetal which is formed by a non enzymatic reaction between GSH and methylglyoxal. Glyoxalase II



catalyses the hydrolysis of this S-D lactoglutathione to D-lactate with the regeneration of GSH in a second step (Singla-Pareek *et al.*, 2003; Kwon *et al.*, 2013).

In plants this pathway has been studied to a limited extent only. *GlyI* gene has been cloned and characterized in some plants species (Deswal and Sopory, 1991; Espartero *et al.*, 1995; Veena *et al.*, 1999; Johansen *et al.*, 2000; Skipsey *et al.*, 2000) and its role in stress tolerance has been indicated (Espartero *et al.*, 1995; Veena *et al.*, 1999; Jain *et al.*, 2002). Many other genes related to salt stress were also introduced in rice by genetic manipulation such as glutamine synthase (GS), choline oxidase (CoD), calcium dependent protein kinase (CDPK), Na<sup>+</sup>/H<sup>+</sup> antiporter and others (Xu *et al.*, 1996; Sakamoto *et al.*, 1998; Tanaka *et al.*, 1999; Hoshida *et al.*, 2000; Saijo *et al.*, 2000; Ohta *et al.*, 2002). The present study was conducted to analyze the integration and expression of *glyI* gene in the next progenies of rice variety PR116 through molecular techniques.

## Materials and Methods

### Strain and plasmid

Plasmid pCAM1304-glyI harboured in E.coli. strain JM109 [Kindly provided by Dr. S.K. Sopory (Jawahar Lal Nehru University, New Delhi)] is a 35S constitutive promoter based plasmid with hygromycin and uidA (GUS) selectable marker genes followed by the nopaline synthase gene (nos) terminator. The source of agency has isolated *glyI* gene from *Brassica juncea* and was cloned in pCAMBIA1304 as NcoI fragment with *hptII* as the selectable marker to get pCAM-glyI. In this case, both *glyI* and the reporter gene *gfp*: *gusA* are driven by a single CaMV35S promoter.

### Genetic transformation through particle bombardment and molecular analysis of T<sub>0</sub> and T<sub>1</sub> progenies of rice variety PR116

Callus induction from the mature seeds were obtained on MS basal medium containing 2, 4-D (2.5 mg<sup>-1</sup>) + Kinetin (0.5 mg<sup>-1</sup>), sucrose (30 g<sup>-1</sup>), proline (560 mg<sup>-1</sup>) + agar (8 g<sup>-1</sup>) and subcultured on callus maintenance medium consisting of MS basal

medium + 2,4-D (2.5 mg<sup>-1</sup>) + kinetin (0.5 mg<sup>-1</sup>) sucrose (30g<sup>-1</sup>) and proline (560 mg<sup>-1</sup>) + agar (30g<sup>-1</sup>). Actively growing embryogenic calli was employed for particle transformation. *Glyoxalase I* was the gene of interest used in this study for increased tolerance towards salinity tolerance. The plate containing target tissue was co-bombarded with tungsten particles coated with plasmid DNA carrying the selectable marker gene (*hpt-II*) and the gene of interest i.e. (*glyI*). Three selection cycles each of 15 days were carried out so as to select hygromycin resistant callus culture. The selected callus were then inoculated on regeneration medium i.e. MS supplemented with BAP (2.0 mg<sup>-1</sup>), kinetin (0.5 mg<sup>-1</sup>), NAA (0.5 mg<sup>-1</sup>), hygromycin (30 mg<sup>-1</sup>). The plantlets regenerated were further put on rooting MS basal medium supplemented with hygromycin (30 mg<sup>-1</sup>). The putative transgenic plants were hardened, kept in the transgenic glass house and maintained at temperature around 30°C with relative humidity 80 percent. Gus analysis of bombarded calli was done. Genomic DNA of each 21 GUS positive transgenic plants of rice variety PR116 were isolated using CTAB method (Doyle and Doyle, 1990). A non transformed plant of the same age rise through tissue culture was used as control. About 50 ng of genomic DNA (3µl) was used as template for each 20µl reaction. PCR mix contained 4µl of 10x PCR buffer, 4 µl of dNTP mix (100 µM), 1 µl of *glyI* gene specific primers containing 1 µM of forward (5'-ATGCGGATGCTGTCCAAGGCG-3') and reverse (5'-TTAAAAGTTATCCTTCGCTCG-3'), 1.2 µl of MgCl<sub>2</sub> (25mM), 1.0 unit of (0.3 µl) Taq DNA polymerase and 5.5 µl of sterile distilled water. PCR conditions used for amplification were initiated with initial denaturation at 94°C for 4 min followed by denaturation at 94°C for 1 min, annealing at 59°C for 2 min and extension for 1min at 72°C, final extension at 72°C for 7 mins. The amplified DNA was analyzed through electrophoresis on 1.0% agarose gels. For the generation advancement and progeny analysis (T<sub>1</sub>), seeds of PCR positive plants were germinated in the glass house. Isolated the DNA of PCR positive plants to check the integration of transgene (*glyI*) through PCR. The same method for DNA isolation and PCR analysis was followed.

**Table 1. Phenotypic differences from methylglyoxal-treated leaf discs of transgenic plants of PR116 (transformed with *Gly I* gene) and non transgenic plant floated in 0, 10 and 15 mM of methylglyoxal solution for 72 h under continuous white light conditions.**

Sr. No.	Name of transgenic line	Phenotypic observation (after 72 h)		
		0 mM (water)	10mM	15mM
1	1-2/10-(1)	Green	Very slight yellow	Late yellow
2	2/5-(1)	Green	Slight yellow	Slight yellow
3	1-2/10-(2)	Green	Very slight yellow	Late yellow
4	2/21	Green	Green	Very slight yellow
5	2/5-(3)	Slight yellow	Slight yellow	Slight yellow
6	Control (non transgenic)	Normal	Complete yellow	Complete yellow

**Table 2. Total chlorophyll content (mg g<sup>-1</sup> fresh weight) from methylglyoxal-treated leaf discs of transgenic plants of PR116 (transformed with *Gly I* gene) and non transgenic plant floated in 0, 10 and 15 mM of methylglyoxal solution for 72 h under continuous white light conditions.**

Sr. No.	Name of transgenic line	Total chlorophyll content (mg g <sup>-1</sup> fresh weight)		
		0 mM (distilled water)	10mM methylglyoxal	15mM methylglyoxal
1	1-2/10-(1)	5.75	2.41	2.69
2	2/5-(1)	7.49	1.69	6.97
3	1-2/10-(2)	4.47	2.77	3.19
4	2/21	10.94	5.69	5.49
5	2/5-(3)	5.57	3.49	3.11
6	Control (non transgenic)	5.77	2.72	2.18

### PCR analysis of T<sub>2</sub> generation of transgenic rice plant

Seeds of 18 PCR positive plants (T<sub>2</sub>) were germinated in the glass house. Isolated the DNA of PCR positive plants to check the integration of transgene (*glyI*) in second generation (T<sub>2</sub>). The same method was followed for DNA isolation and PCR analysis in this generation.

### PCR analysis of T<sub>3</sub> generation of transgenic rice plant

In the next generation, seeds of 11 PCR positive plants were germinated in the glass house. Isolated the DNA of 11 PCR positive plants to check the

integration of transgene (*glyI*). PCR analysis was conducted to check the integration of gene through specific designed primers.

### Leaf disc assay for tolerance against MG in T<sub>3</sub> generation

This experiment was carried out on the selected T<sub>3</sub> transgenic lines (PCR positive) tested for enzyme activity. All the PCR positive plants were tested for methylglyoxal sensitivity in T<sub>3</sub> generation. For this purpose, leaf disc were floated in 10 and 15 mM solution of methylglyoxal for 72 hr. leaf disc floated in water served as experimental control. Figure shows the phenotypic differences among transgenic and non transgenic plants after 72 h of MG treatment.

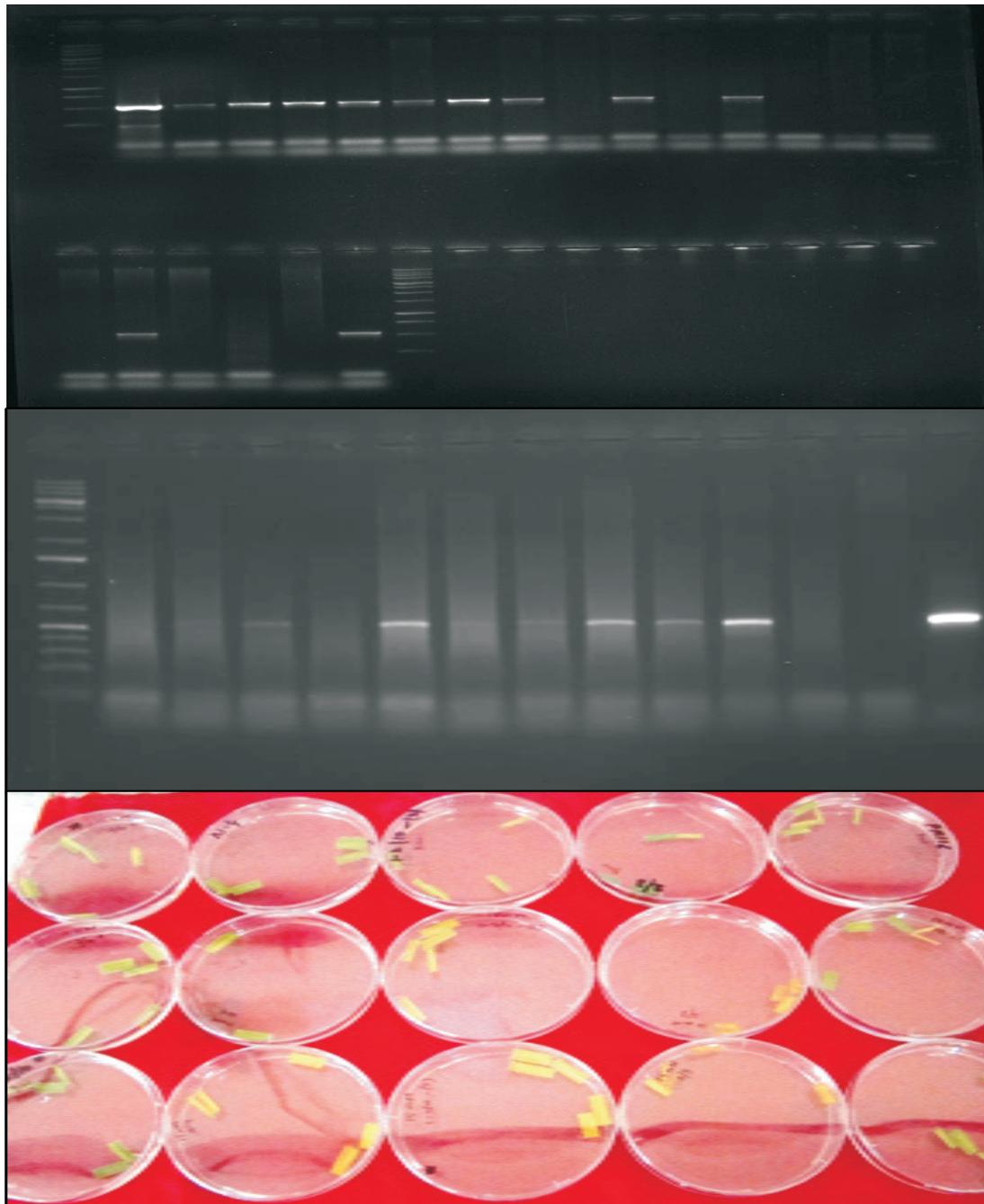


Figure 1. Progeny analysis of transgenic rice variety PR116 transformed with Glyoxalase I gene

- (a) PCR analysis of second generation ( $T_2$ ) of transgenic plants of *Indica* rice variety PR116, M- 100bp DNA Ladder, 1-18: transgenic DNA, 19- non transgenic (negative control), 20- water (negative control), 21- plasmid DNA (positive control)
- (b) PCR analysis of third generation ( $T_3$ ) of transgenic plants of *Indica* rice variety PR116, M- 100bp DNA Ladder, 1-11: transgenic DNA, 12- non transgenic (negative control), 13- plasmid DNA (positive control)
- (c) Representative image showing phenotypic differences from methylglyoxal-treated leaf discs of transgenics and non transgenic plants after incubation in 0, 10, 15mM solutions for 72h are shown. Discs floated in water served as the experimental control

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The chlorophyll content was measured as described (Arnon, 1949). The experiment was repeated thrice with three different transgenic lines.

## Results and Discussion

### Development of transgenic rice plants and molecular analysis

The embryogenic calli were placed in about 1cm diameter on the osmoticum medium prior to 4hr of particle gun mediated transformation. After bombardment, the callus cultures were maintained on MS medium containing 2.5 mg/l 2, 4-D + 0.5 mg/l kinetin + sucrose (30 g/l) along with hygromycin (30 mg/l) selection. Out of the 2620 bombarded embryogenic calli 266 calli were selected on selective medium and percent selection recorded was 10.15. The selected calli were incubated in X-gluc solution at 37°C in the total dark to know the frequency of GUS expression. Clearly shows the appearance of the blue sections in the calli was taken as indicator of gusA expression in the bombarded calli which was recorded to be 47.50%. Out of the 266 selected calli 21 showed regeneration and percent regeneration was 7.89. It took 6 week to regenerate complete shoots after incubation on shoot proliferation medium supplemented with hygromycin. Following bombardment of 2620 embryogenic calli, 21 independently regenerated rice plants were obtained on hygromycin selection and vigourously growing putative transgenic lines with robust root system on half strength MS basal medium and were successfully transplanted in the glasshouse.

The regenerated putative transgenics plantlets appeared normal phenotypically. A total of 21 gus positive plants were analysed by PCR for the presence of *gyl1* gene (576 bp) using specific primer pairs. PCR analysis of genomic DNA from all the 21 transgenic lines, 7 revealed the presence of 576 bp band for the transgene (*gyl1*). Each plant had 3-4

copies in next generation ( $T_1$ ). PCR analysis of total 21 PCR (3-4 copies of 7 PCR positive plants) positive  $T_1$  generation confirmed the presence of *Glyoxalase I* of 576 bp in 18 plants. PCR positive plants were grown and harvested seeds ( $T_2$ ). In the next generation ( $T_2$ ), seeds of 18 PCR positive plants were grown in the pots. The regenerated transgenic plants appeared normal phenotypically. PCR analysis confirmed integration of gene (*gyl1*) in 11 plants of variety PR116. Seeds of 11 PCR positive plants were collected and grown in the next ( $T_3$ ) generation to check the integration and expression of *gyl1* gene. Plants which showed integration of *glyoxalaseI* gene with specific primers in last generation were tested for methylglyoxal sensitivity.

This experiment was carried out on the selected transgenic lines tested for enzyme activity. All the PCR positive plants were tested for methylglyoxal sensitivity in  $T_3$  generation. The leaf disc floated in 10 and 15 mM solution of methylglyoxal for 72 hr showed the phenotypic differences among transgenic and non transgenic plants after 72 h of MG treatment. Leaf disc floated in water served as experimental control (Figure 1). Based on visual observations, it was found that transgenics plant tolerated MG at higher concentrations than non transgenic plant (Figure 1, Table 1 and 2). Measurement of chlorophyll content indicated that the leaf discs of the some transgenics retained more chlorophyll, whereas the WT plants retained only very less chlorophyll in 15 mM (Table 2). The ability of the transgenic plants to maintain physiological levels of chlorophyll under different methylglyoxal concentration (stress condition) was level for the measurement of injury caused by the stress. At 10 or 15 mM MG, the transgenics performed better (i.e., they were able to maintain higher levels of chlorophyll contents in the tissue subjected to stress, Table 2) than the non transformed lines. Out of all the transgenic lines, 2/21 showed higher chlorophyll content at the level of 10 and 15 mM concentrations (5.69 and 5.49 mg g<sup>-1</sup> fresh weight of total chlorophyll) whereas the non transgenic line showed minimum chlorophyll (2.18 mg g<sup>-1</sup>) content at the level of 15mM.



The similar study was also reported by Veena *et al.* (1999) that transgenic plants overexpressing *glyoxalase I* showed significant tolerance to methylglyoxal and high salt as tested in detached leaf disc senescence assay. Singla-Pareek *et al.* (2003) observed in transgenic rice plants and reported that glyoxalase II that increased further upon salt stress, reflecting the upregulation of endogenous glyoxalase II. The transgenic rice showed higher tolerance to toxic concentration of methylglyoxal (MG) and NaCl compared with non transgenic, transgenic plants at the T<sub>1</sub> generation exhibited sustained growth and more favorable ion balance under salt stress conditions. Verma *et al.* (2003) studied the expression of *glyI* gene and observed phenotypic difference between transgenic and non transgenic plants after methylglyoxal treatment. Non transgenic plants showed early bleaching senescence compared with transgenic plants harboring *glyoxalase I* gene on different concentrations of methylglyoxal. The non transgenic plants showed decrease in chlorophyll content was 25%, 35% and 60% at 200mM, 400mM and 800mM NaCl concentrations. While in transgenic lines no such sharp fall in chlorophyll content was noticed. In response to methylglyoxal treatment wild type plants also showed sharp declines in chlorophyll content with increasing concentration of methylglyoxal as compared to the transgenic plants. Yadav *et al.* (2005) studied the expression of *glyI* in tobacco and observed that the level of methylglyoxal did not increase in response to stress compared to the untransformed plants. Integration and expression analysis of *glyII* gene in generations through polymerase chain reaction, southern blotting and salinity tolerance of transgenic plants were studied by Saxena *et al.* (2011) in *Brassica*. The transgenic plants transformed with *glyoxalase II* gene showed higher salinity tolerance as compared to the untransformed control plants. The percent germination of the T<sub>2</sub> transgenic seeds was higher at 150mM and 200mM NaCl as compared to the seeds of transformed plants.

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