17. Overexpression of *Glyoxalase I* gene confers salinity tolerance in transgenic *japonica* and *indica* rice plants

M. VERMA[1,2], D. VERMA[1,2], R. K. JAIN[2], S. K. SOPORY[3] and R. WU[1,4]

1) Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY, 14853 USA

2) Department of Biotechnology and Molecular Biology, Haryana Agricultural University, Hisar-125004 India

3) International Centre for Genetic Engineering and Biotechnology, New Delhi, 110067 India

4) Corresponding author: E-mail: ray.wu@cornell.edu

Salinity limits growth and productivity of all major crops including rice. At present approximately 20% of cultivated land and nearly half of all irrigated lands are affected by salinity. As a result of high salt concentrations, ionic imbalance and hyper-osmotic stress are triggered in plants, which consequently elicit secondary stresses such as oxidative damage (Zhu 2001). Several studies have shown that numerous enzymes/proteins help in the removal of the cytotoxic and cell growth inhibitor compounds. This leads to increased cell growth and tolerance to stress (Zhang et al. 2000). The glyoxalase system has been proposed to be involved in various functions in animal systems, which include regulation of cell division and proliferation, microtubule assembly, and protection against oxoaldehyde toxicity (Thornalley 1990). Glyoxalase enzymes are important for the glutathione (GSH)–based detoxification of methylglyoxal, which is formed primarily as a byproduct of carbohydrate and lipid metabolism. Methylglyoxal is a potent mutagenic and cytotoxic compound known to arrest growth, react with DNA and proteins, and increase sister chromatid exchange. A cell producing more glyoxalase could convert increased amounts of methylglyoxal into a non-toxic form, thus protecting the cell from the methylglyoxal cytotoxic and mutagenic effects. Singla-Pareek et al. (2003) genetically engineered the glyoxalase pathway in tobacco leading to enhanced salinity tolerance.

In this study, *glyoxalase I* gene of *Brassica juncea* (Veena et al. 1999), driven by a stress-inducible ABRC1-Act1-100P-HVA22 (I) promoter complex and flanked by tobacco *Rb7* MAR sequences (Fig. 1), was biolistically transferred into scutellum-derived, three-week-old calli of japonica rice (*Oryza sativa* L.) variety TNG67 and indica rice variety Pusa Basmati 1 using the protocol of Cao et al. (1992). The *bar* cassette containing *phosphinothricin acetyl transferase* gene controlled by CaMV 35S promoter and *nos* 3' region served as the selectable marker gene.

MAR::ABA I (P)::*glyoxalase I*::Pin 3'::MAR::35S (P)::*bar*::*nos* 3'

Fig. 1. Physical map of plasmid pSB5 (6.3 kb). A unique *Cla*I site is located between MAR and ABA I (P). MAR (1.1 kb), tobacco *Rb7* matrix attachment region sequences; ABA I (P) (0.6 kb), an ABA-inducible promoter, which is comprised of ABRC1-Act1-100P-HVA22 I complex (Su et al. 1998); *glyoxalase I* (0.8 kb), gene from *Brassica juncea*; Pin 3' (1.0 kb), *Pin2* 3' region; 35S (P) (0.8 kb), cauliflower mosaic virus 35S promoter; *bar* (0.6 kb), *phosphinothricin acetyl transferase* gene; *nos* 3' (0.3 kb), *nopaline synthase* gene 3' region.

A total of 500 and 200 bombarded embryogenic calli of TNG67 and Pusa Basmati 1 were respectively cultured every 3 weeks onto callus induction medium containing Bialaphos (6 mg/l) in the dark to minimize the number of escapes. After 6 weeks, on average, 40-48% calli showed sustained growth and cell proliferation in the selection medium. Nearly 71% of resistant TNG67 calli regenerated one or more shoots when transferred onto regeneration medium. Forty-one percent of Pusa Basmati 1 calli regenerated into plants. These regenerated plants were successfully transplanted into pots and grown to maturity in the greenhouse. Fertile transgenic rice plants were produced with transformation frequency varying between 8 and 10%.

Preliminary screening of putative transgenic rice plants was conducted using herbicide BASTA™ resistance test. For Basta^R test, a water solution containing 0.35% (v/v) commercial herbicide Basta (Hoechest-Roussel Agro-vet company, Somerville, NJ) for TNG67 and 0.2% (v/v) for Pusa Basmati 1, mixed with 0.1% (v/v) Tween-20, was painted on both sides of a leaf. After 7 days, the resistant/sensitive phenotypes were scored. The leaves in >80% of transgenic plants remained green or showed little or no discoloration in resistant plants, but turned yellow in sensitive plants. PCR analysis using glyoxalase-I-specific primers showed amplification of the expected 572-bp fragment in over 95% of Bialaphos-resistant transgenic R_0 plants of the two rice varieties (data not shown). DNA from the PCR-positive plants was subjected to Southern blot hybridization to confirm stable integrative transformation with respect to the glyoxalase I gene.

Genomic DNA was digested with *Eco*RI enzyme to release the glyoxalase I cDNA fragment. A common band of expected size, 800 bp, was detected by DNA blot analysis in TNG67 transgenic lines (data not shown). In addition, restriction digestion of genomic DNA with *Cla*I (unique site in plasmid SB5) enzyme from the selected transgenic plants resulted in the appearance of hybridization bands at different positions, suggesting that transgenic lines were produced from different transformation events (Fig. 2 A and B). The number of bands indicated the copy number. The hybridization signals indicated

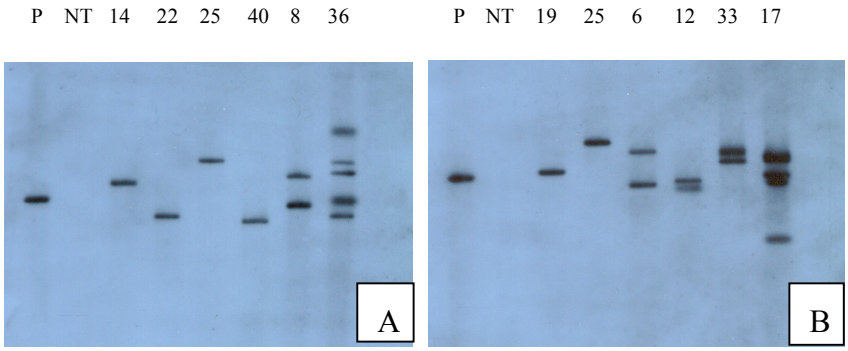

Fig. 2. DNA blot analysis of six representative R_0 TNG67 (A) and Pusa Basmati 1 (B) plants where plant genomic DNA was digested with *Cla*I and hybridized with DIG-labeled 572-bp DNA fragment containing Glyoxalase I coding region. P, Plasmid DNA**;** NT**,** non-transgenic rice plant.

that four TNG67 (Fig. 2A) and two Pusa Basmati 1 plants (Fig. 2B) had a single copy of the transgene with a frequency of 9% and 7%, respectively. Results of DNA blot hybridization were generally consistent with those of the herbicide BASTA™ resistance test, suggesting that both the selectable marker gene and the glyoxalase I gene on the same plasmid were efficiently co-integrated into the rice genome. The transgenic rice plants were morphologically normal. This is probably due to the use of a stress-inducible promoter to drive the expression of the transgene, which produces the glyoxalase I protein only under stress conditions. Transgenic plants were analyzed for methylglyoxal and salt sensitivities. Healthy and fully expanded leaves from wild-type and transgenic rice plants were detached and washed briefly in deionized water. Leaf discs of 1 cm diameter were cut and floated on 5 ml of methylglyoxal (5 and 20 mM) and sodium chloride (200, 400 and 800 mM) solutions for 24 h and 72 h, respectively, by allowing the upper surface of the leaf to be in contact with the solution. The treatment was carried in continuous white light at 25±1°C. The leaf discs were extracted with DMF (N, N′-dimethyl formamide), and the chlorophyll content was measured as described by Porra et al. (1989). Phenotypic differences were observed between non-transgenic and transgenic plants after treatment. Non-transgenic plants showed early bleaching/senescence compared with transgenic plants harboring glyoxalase I gene. Measurement of chlorophyll content in the leaf discs of these plants further confirmed the observed phenotypic differences in the treated leaf discs as shown in Fig. 3. The results showed that in Pusa Basmati 1 wild-type plants decrease in chlorophyll content was 25%, 35% and 60% at 200 mM, 400 mM and 800 mM NaCl concentrations, respectively (Fig. 3A Bar #1), while in transgenic lines no such sharp fall in chlorophyll content was noticed (Fig. 3A Bar # 2, 3 & 4). A similar trend was observed in TNG67 where decrease in chlorophyll content in wild-type plants was 20%, 28% and 45% at 200 mM, 400 mM and 800 mM NaCl

concentrations, respectively (Fig. 3A Bar # 5), and transgenic lines showed no such severe drop in chlorophyll content (Fig. 3A Bar # 6, 7 & 8). In response to methylglyoxal treatment wild-type plants also showed sharp declines in chlorophyll content with increasing concentrations of methylglyoxal (Fig. 3B Bar # 1 & 5) as compared to the transgenic plants (Fig. 3B Bar # 2, 3, 4, 6, 7 & 8).

Finally, at all the concentrations of NaCl and methylglyoxal tested, we found that the decrease in the chlorophyll content in transgenic plants was less compared with the wild-type plants, and the resistance against stress was clearly indicated by delayed senescence and the presence of high chlorophyll content in the leaf discs obtained from sense transgenic plants. The T_0 plants grew normally and set seeds. These findings suggest that stress induced overexpression of *glyoxalase I* gene in transgenic rice plants can confer increased tolerance under salt stress.

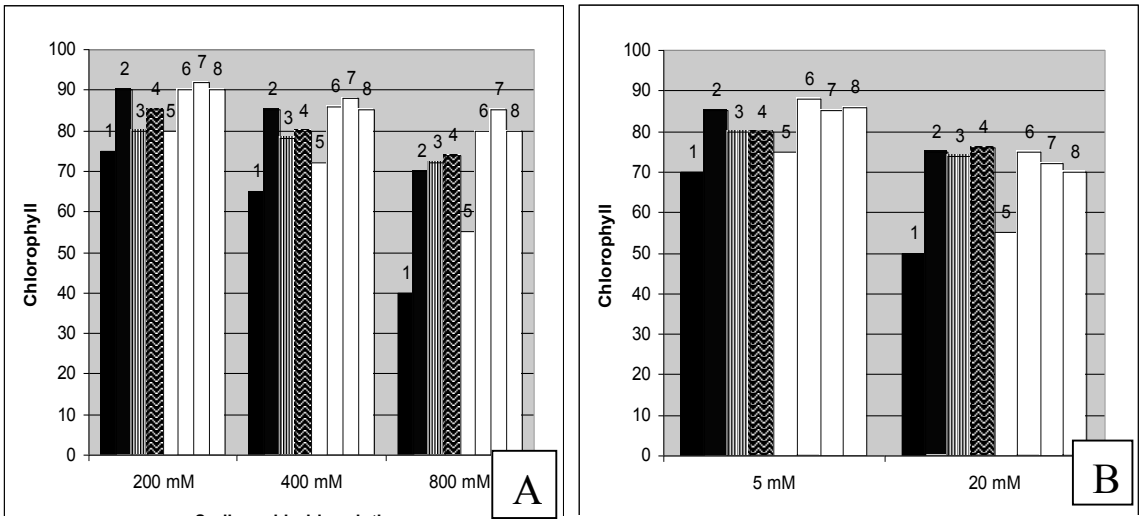

Fig. 3. Chlorophyll content in transgenic lines harboring *glyoxalase I* gene and wild-type leaf discs after 72 hours of treatment on NaCl solution (A) and methylglyoxal solution (B). Chlorophyll content in plants in the absence of salt treatment was determined and set at 100. The results shown in the bar graph are values relative to samples without stress. Bar 1 represents Pusa Basmati 1 Control; Bar 2, 3, 4 represent R_0 Transgenic Pusa Basmati 1 line # 19, 25, 17, respectively; Bar 5 represents TNG67 Control; Bar 6, 7, 8 represent R_0 Transgenic TNG67 line # 14, 25, 36, respectively.

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References

Cao, J., X. Duan, D. McElroy and R. Wu, 1992. Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. Plant Cell Rep. 11: 586-591.

Porra, R. J., W. A. Thompson and P. E. Kreidemann, 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim. Biophys. Acta 975: 384-394.

Singla-Pareek, S. L., M. K. Reddy and S. K. Sopory, 2003. Genetic engineering of the glyoxalase pathway in tobacco leads to enhanced salinity tolerance. Proc. Natl. Acad. Sci. USA 100: 14672-14677.

Su, J., Q. Shen, T.-H. D. Ho and R. Wu, 1998. Dehydration-stress-regulated transgene expression in stably transformed rice plants. Plant Physiol. 117: 913-922.

Thornalley, P. J., 1990. The glyoxalase system: new developments towards functional characterization of metabolic pathways fundamental to biological life. Biochem. J. 269: 1-11.

Veena, V. S. Reddy and S. K. Sopory, 1999. Glyoxalase I from *Brassica juncea:* molecular cloning, regulation and its over-expression confer tolerance in transgenic tobacco under stress. Plant J. 17: 385-395.

Zhang, J., N. Y. Klueva, Z. Wang, R. Wu, T.-H. D. Ho and H. T. Nguyen, 2000. Genetic engineering for abiotic stress resistance in crop plants. In Vitro Cell Dev. Biol. Plant 36: 108-114.

Zhu, J. K., 2001. Plant salt tolerance. Trends Plant Sci. 6: 835-840.