BIOLOGIA PLANTARUM 55 (3): 536-540, 2011

BRIEF COMMUNICATION

Introduction of *OsglyII* gene into *Oryza sativa* for increasing salinity tolerance

S.H. WANI[1]* and S.S. GOSAL[2]

Department of Plant Breeding and Genetics PAU, Ludhiana 141004, India[1]
School of Agricultural Biotechnology PAU, Ludhiana 141004, India[2]

Abstract

Mature seed-derived embryogenic calli of *indica* rice (*Oryza sativa* L. cv. PAU201) were induced on semisolid Murashige and Skoog medium supplemented with 2.5 mg dm^{-3} 2,4-dichlorophenoxyacetic acid + 0.5 mg dm^{-3} kinetin + 560 mg dm^{-3} proline + 30 g dm^{-3} sucrose + 8 g dm^{-3} agar. Using *OsglyII* gene, out of 3180 calli bombarded, 32 plants were regenerated on medium containing hygromycin (30 mg dm^{-3}). Histochemical GUS assay of the hygromycin selected calli revealed GUS expression in 50 % calli. Among the regenerants, 46.87 % were GUS positive. PCR analysis confirmed the presence of the transgene of 1 kb in 60 % of independent plants. Further, these plants have been grown to maturity in glasshouse. *In vitro* screening for salt tolerance showed increase in fresh mass of *OsglyII* putative transgenic calli (185.4 mg) as compared to control calli (84.2 mg) on 90 mM NaCl after 15 d. When exposed to 150 mM NaCl, *OsglyII* putative transgenic plantlets showed normal growth while the non-transgenic control plantlets turned yellow and finally did not survive.

Additional key words: embryonic callus, growth regulators, GUS expression, hygromycin, proline, rice.

Rice (*Oryza sativa* L.) is more susceptible to damage from water deficit as compared to other cereals (Lafitte and Bennet 2003) and salinity is widespread soil problem in rice-growing countries. Breeding for salt tolerance in rice has been difficult task due to the involvement of several genes and insufficient knowledge about mechanism(s) controlling this character (Akita and Cabuslay 1988, Yeo *et al.* 1990).

For many decades, it is known that the glyoxalase system occurs in animals, however few studies have been carried out so far to understand the glyoxalate pathway in plants. Cloning and characterization of *glyoxalase I* has been reported from a few plants (Deswal and Sopory 1991, Espartero *et al.* 1995, Veena Reddy *et al.*1999, Johansen *et al.* 2000, Skipsey *et al.* 2000). Glyoxalate pathway has been linked with rapidly dividing plant cells (Deswal *et al*. 1993, Paulus *et al*. 1993) and has been reported to be involved in stress tolerance (Espartero *et al.* 1995, Veena Reddy *et al.* 1999, Jain *et al.* 2002).

Abiotic stresses have been shown to have a quantitative character, and thus they are controlled by multiple genes (Hasegawa *et al.* 2000). However, there are number of instances where single-gene transfers have led to the development of tolerant plants (for review see Singh *et al.* 2008, Gosal *et al.* 2009). The first report of transgenic plants produced for abiotic stress tolerance was in tobacco (Tarczynski *et al.* 1992) using *mtlD* gene for mannitol-1-phosphate dehydrogenase from *E. coli*. The role of *Osgly II* gene for imparting tolerance against salinity has been earlier reported in tobacco (Singla-

Received 23 October 2009, *accepted* 15 February 2010.

Abbreviations: BAP - benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; GUS - β-glucoronidase; Kin - kinetin; NAA - napthaleneacetic acid; *Osgly II* - *Oryza sativa glyoxalase II*; PDS - particle delivery system; SLG - SD-lactoylglutathione; x-gluc - 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexylammonium salt.

Acknowledgement: We are grateful to Prof. Sudhir Kumar Sopory, Group Leader, Plant Molecular Biology, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg110 067, New Delhi, India, for kindly providing the *Osgly II* gene construct and Department of Biotechnology, Govt.of India, New Delhi for funding support.

* Corresponding author, fax: (+91) 0194 2305045, e-mail: shabirhussainwani@gmail.com

Pareekh *et al.* 2003) and rice (Singla-Pareekh *et al.* 2008) but, in both the cases the transformation was *Agrobacterium* mediated. Here, we report for the first time a particle gun mediated introduction of *Osgly II* gene into high yielding *indica* rice.

Seeds of *indica* rice (*Oryza sativa* L. cv. PAU 201) were provided by the Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana, India. Manually dehusked seeds were treated with 0.1 % *Bavistin* for 3 h followed by washing thrice with distilled water. These seeds were then surface sterilized in 0.1 % $HgCl_2$ for 8 - 10 min followed by three rinses with autoclaved distilled water. Sterilized seeds were cultured on semisolid Murashige and Skoog (1962; MS) medium supplemented with 2.5 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) + 0.5 mg dm^{-3} kinetin (Kin) + 560 mg dm^{-3} proline + 30 g dm^{-3} sucrose + 8 g dm^{-3} agar. After two weeks of incubation, the embryogenic scutellar calli (2 - 3 mm in diameter) were excised from the germinating seeds and arranged in 1 cm circle (20 to 25 calli) in the center of the target plate (90 × 15 mm) containing osmoticum (Fig. 1).

OsglyII gene construct was kindly provided by Dr. S.K. Sopory (ICGEB, New Delhi India). The details of the gene construct are given in Singla-Pareekh *et al*. 2003. Bombardment of embryogenic calli was performed using *1000 He* gun system (*Bio Rad*, Hercules, USA). Tungsten particles (0.7 μm) coated with DNA were accelerated towards the tissue placed at 9 cm away from macrocarrier launch assembly with rupture discs withstanding 1100 psi pressure. After bombardment the Petri dishes were sealed with parafilm and incubated at 25 ± 2 ºC under diffused light. After 16 h the bombarded calli were transferred onto above mentioned MS medium for 2 d. The calli were then subcultured on the selection medium containing hygromycin (30 mg dm^{-3}) for two cycles of 2 weeks each and the resistant calli were regenerated into plantlets (Fig. 1). Putative transgenic calli were transferred to shoot regeneration MS medium supplemented with benzylaminopurine (BAP; 2.0 mg dm^{-3}) + Kin (0.5 mg dm^{-3}) + naphthaleneacetic acid (NAA; 0.5 mg dm^{-3}) + hygromycin (30 mg dm^{-3}) + sucrose (30 g dm^{-3}) + agar (8 g dm^{-3}). After 2 weeks, the surviving calli were again transferred to fresh shoot regeneration medium. Regenerated plantlets were rooted on basal MS medium + hygromycin (30 mg dm^{-3}). Putative transgenic plantlets were hardened by placing them on absorbent cotton in the test tubes initially for 1 week where the plantlets resumed their growth. Plantlets were then directly kept in test tubes in the tap water for another week where profuse rooting occurred and the plantlets got ready for transferring to the soil. Hardened plantlets were finally transferred to the soil in the plastic pots and were kept in transgenic glasshouse maintained at 30 ºC with 80 % relative humidity (Fig. 1).

In vitro screening of non-transgenic calli was carried out to check the inherent tolerance of calli for salt stress using NaCl in six concentrations (0, 30, 60, 90, 120 and 150 mM) added to the callus induction medium. Equal quantities (100 mg) of one month old embryogenic calli were subcultured on medium containing different concentrations of NaCl. Bombarded calli after selection on medium containing hygromycin (30 mg dm^{-3}) were grown on 90 mM NaCl along with control calli for 2 weeks and increase in fresh mass was recorded. After hardening, the plantlets were transferred to soil. One week after their transfer, putative transgenic plants were screened for salt tolerance by keeping them in MS liquid medium supplemented with 150 mM NaCl for 15 d. Putative transformants were screened by PCR analysis using rice genomic DNA from non-transformed and putative transgenic plants as template and *OsglyII* forward (5′-ATGCGGATGCTGTCCAAGGCG-3′) and reverse (5′-TTAAAAGTTATCCTTCGCTCG-3′) primers as described earlier (Singla-Pareekh *et al.* 2003). The PCR was carried out in 0.02 cm^3 solution comprising 50 ng of rice genomic DNA, 0.004 cm^3 of 5× PCR buffer (*Promega*, Madison, WI, USA) (pH 8.5), 1.2 cm^3 of 25 mM $MgCl_2$, 3.0 cm^3 of 1.0 mM dNTP mix, 1 U Taq DNA polymerase and 1 cm^3 of 20 μM of each primer. The PCR profile was an initial denaturation at 94 ºC for 4 min followed by 40 cycles of denaturation at 94 ºC for 1 min, primer annealing at 57 ºC for 2 min and enzymatic extension at 72 ºC for 1 min and final extension at 72 ºC for 7 min followed by rapid cooling at 4 °C.

For transformation, a total 10 experiments were carried out and a total 3180 calli were bombarded with *OsglyII* gene. After two cycles of selection, 425 calli were selected which were capable of growing on selection medium containing hygromycin (Table 1). Further, 408 calli were transferred to regeneration medium in the presence of hygromycin and were incubated under irradiance of 67.5 μmol m^{-2} s^{-1}.

Hygromycin has been found to be safe and effective selecting agent for transgenic tissues in rice (Christou

Table 1. Genetic transformation of rice cv. PAU 201 by bombarding scutellar-derived calli with *OsglyII* gene.

Exp. No.	Number. of calli bomb.	Number of calli selected	Number of calli on regeneration medium	Number of regenerated plantlets
1	300	40	38	4
2	320	38	36	2
3	360	56	55	4
4	400	54	52	4
5	300	44	43	4
6	320	40	38	2
7	300	36	34	2
8	240	30	29	1
9	300	42	40	5
10	340	45	43	4
Total	3180	425	408	32

et al. 1991, Maqbool *et al.* 1998, Oszvald *et al.* 2008, Singla-Pareek *et al.* 2008). Different rice cultivars possess different sensitivity to hygromycin. Generally, concentrations ranging from 25 mg dm^{-3} to 50 mg dm^{-3} have been found to inhibit the growth of non-transformed tissues. After 3 weeks of incubation, some of the calli exhibited the development of green spots and shoot primordia along with the regeneration of shoots. Complete plantlets were developed after 5 weeks of incubation. A total 32 independent putative transgenic plantlets were regenerated (Fig. 1*L*). The transformation efficiency through particle bombardment is low as compared to *Agrobacterium*-mediated transformation. Among various factors responsible, one observed in our laboratory was poor entry of tungsten particles into the target tissue. When analyzing the bombarded tissue under electron microscope, we observed that most of the DNA coated tungsten particles did not enter the tissue. Later, this problem was overcome by adjusting the distance between the target plate and macrocarrier launch assembly. In some instances, the subcultured calli turned necrotic (brown). Browning of subcultured calli of rice has been the recurring problem (Mitsuoka *et al.* 1994, Hoque and Mansfield 2004). Randomly selected calli portions were GUS assayed and GUS expression (blue spots) was shown in 50 % calli (Fig. 1*C*) whereas the non-transformed (control) did not develop any blue colour even after incubation in the x-gluc solution. A total of 32 independently regenerated plantlets were tested, of which 15 (46.87 %) were found to be GUS positive. These 15 T_0 plants were hardened and transferred to soil in the earthen pots and grown in glasshouse. They exhibited normal growth and flowering (Fig. 1*O*). PCR analysis confirmed the presence of transgene of 1 kb in 9 out of 15 (60 %) independent plants when total genomic DNA from various independent putative

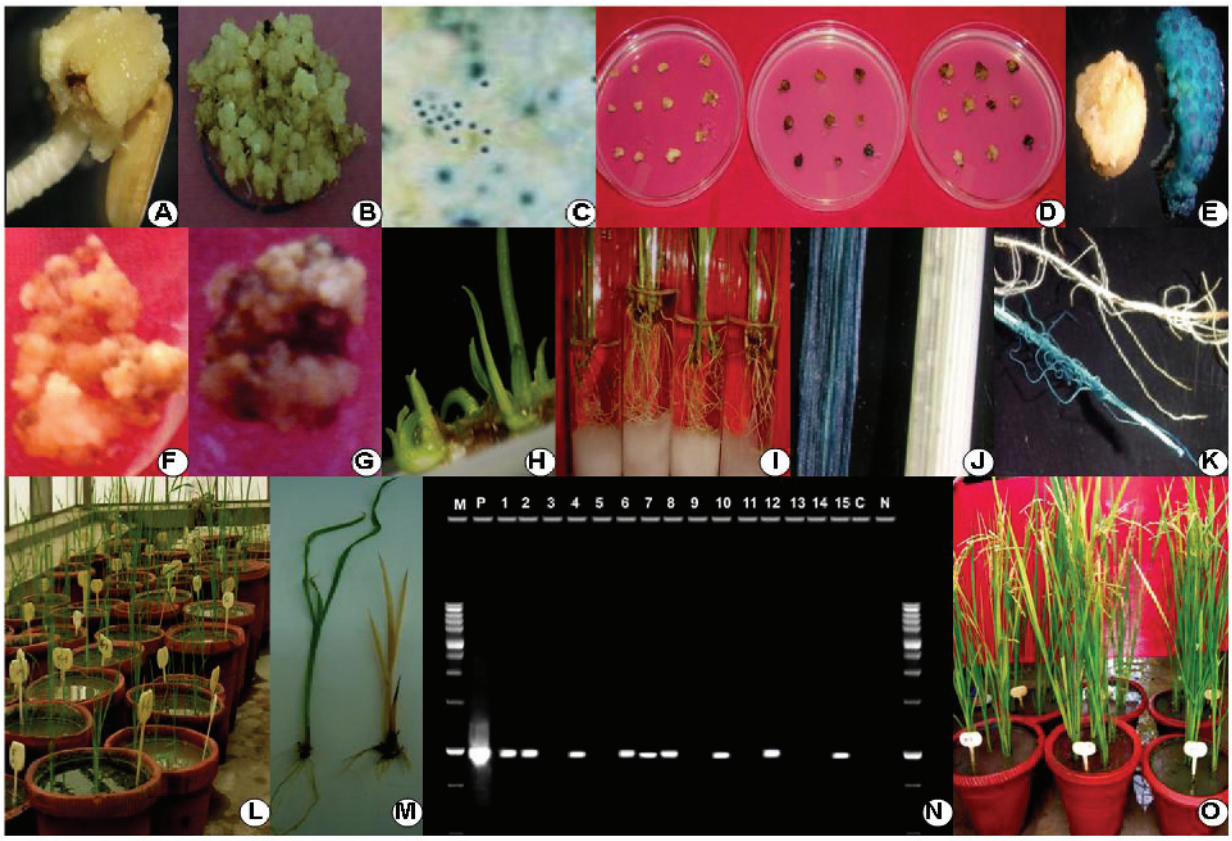

Fig. 1. Scuteller-derived embryogenic callus with seed (*A*). Target plate used for bombardment (*B*). Stereoscopic view of bombarded calli 48 h after bombardment (*C*). Selection of bombarded calli: non-transformed calli on medium without hygromycin (*left*), non-transformed calli on medium containing hygromycin (*middle*) and transformed calli on medium containing hygromycin (*right*) (*D*). Transient GUS expression after 1 week of selection (*E*). *OsglyII* transgenic calli showing increased fresh mass on medium containing 90 mM NaCl (*F*). Control calli showing necrosis and decrease in fresh mass on medium containing 90 mM NaCl (*G*). Shoot differentiation from bombarded calli (*H*). Rooting of putative transgenic plantlets (*I*). Stereoscopic view of T_0 rice leaf cutting showing GUS expression and control leaf (*J*). Stereoscopic view of T_0 rice root cuttings showing GUS expression and control root cuttings (*K*). Population of Os*gly II* putative transgenic plants in glasshouse (*L*). *OsglyII* transgenic and control plantlets after a shock of 150 mM NaCl for 15 d in MS liquid medium (*M*). PCR amplification of Os*glyII* in transgenic rice plants (N). Putative transgenic plants grown in the earthen pots showing normal flowering (*O*).

transgenic plants was used as the template and end sequences of *OsglyII* as the primers in the PCR analysis (Fig. 1*N*). Singla-Pareekh *et al.* (2008) reported PCR analysis of transgenic rice plants expressing *OsglyII* gene and all the 30 independent lines were found PCR positive.

In vitro screening of salt tolerance was carried out at calli and plantlet level using NaCl as stress agent. Seeds of *Salicornia persica* and *S. europaea* were germinated under NaCl (0, 100, 200, 300, 400, 500, and 600 mM) on MS medium for 45 d (Aghaleh *et al*. 2009). Similarly, NaCl (0, 250 and 500 mM) were used to create salinity effect on 10-d-old seedlings of wheat (Mutlu *et al.* 2009). Two wheat cultivars, Mahon Demias and Hidhab exhibited appreciable callus induction but differed significantly in the capacity of calli proliferation and regeneration under salinity stress (Benderradji *et al.* 2007). *In vitro* studies on salt tolerance were also carried out with two *indica* rice cultivars, CSR27 (salt tolerant) and HBC19 (salt sensitive). Calli were transferred to MS medium containing 0 (control), 0.5, 1.0, 1.5 and 2.0 % NaCl (m/v) for four weeks. Seedlings (14-d-old) were also exposed to salt treatment in half-strength MS salts solution with 0 (control), 0.25, 0.50, 0.75, 1.0 and 1.50 % NaCl for one week. High salt concentration decreased the relative growth, based on fresh mass of both callus and seedlings in rice (Thach and Pant 1999). In our case, the calli grown on control medium exhibited normal proliferation. As the concentration of NaCl in the medium increased, fresh mass of callus decreased. After 2 weeks at 90 mM NaCl, the fresh mass of calli was 111.23 mg as compared to 194.24 mg at 0 mM (42.7 % reduction). Therefore, 90 mM NaCl was taken as standard for screening the putative transgenic calli. *OsglyII* bombarded calli were put for selection on medium containing hygromycin for 2 weeks. Thereafter, surviving calli (approximately 100 mg) were exposed to 90 mM NaCl for 2 weeks. Callus fresh mass of transgenic calli at 90 mM NaCl was 185.47 mg while it remained only 84.23 mg in case of control calli. Plant regeneration of putative *OsglyII* transgenic calli at 90 mM NaCl was 15.67 % while 7.77 % in case of control calli. Further, putative *OsglyII* transgenic calli showed normal growth on MS medium supplemented with 150 mM NaCl, whereas controls showed reduced growth browning and finally died (Fig. 1*F*,*G*).

Our results indicate that *OsglyII* transgenic plants showed tolerance to salt stress both at calli and plantlet level. Similar results have been demonstrated in rice (Su *et al.* 2006) and tobacco (Singla-Pareekh *et al.* 2003).

References

Aghaleh, M., Niknam, V., Ebrahimzadeh, H., Razavi, K.: Salt stress effects on growth, pigments, proteins and lipid peroxidation in *Salicornia persica* and *S. europaea*. - Biol. Plant. **53**: 243-248, 2009.

Akita, S., Cabuslay, G.S.: Physiological basis of differential response to salinity in rice lands. - In: Proc. 3rd Int. Symp. Genet. Aspects Plant Mineral Nutr. Pp. 37. Braunschweig 1988.

Benderradji, L., Bouzerzou, H., Djekoun, A., Yekhlef, N., Benmahammed, A.: Effects of NaCl stress on callus proliferation and plant regeneration from mature embryos of bread wheat (*Triticum aestivum* L.) cultivars Mahon demias and Hidhab. - Plant Tissue Cult. Biotechnol. **17**: 19-27, 2007.

Christou, P., Ford, T.L., Kofron, M.: Production of transgenic rice (*Oryza sativa L.*) plants from agronomically important *Indica* and *Japonica* varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. - Bio/Technology **9**: 957-962, 1991.

Deswal, R., Sopory, S.K.: Purification and partial character-rization of glyoxalase I from a higher plant *B. juncea*. - FEBS Lett. **282**: 277-280, 1991.

Deswal, R., Chakravarty, T.N., Sopory, S.K.: The glyoxalase system in higher plants: regulation in growth and differentiation. - Biochem. Soc. Trans. **21**: 527-530, 1993.

Espartero, J., Sanchez-Aguayo, I., Pardo, J.M.: Molecular characterization of glyoxalase I from a higher plant: upregulation by stress. - Plant mol. Biol. **29**, 1223-1233, 1995.

Gosal, S.S., Wani, S.H., Kang, M.S.: Biotechnology and drought tolerance. - J. Crop Improv. **23**: 19-54, 2009.

Hasegawa, P.M., Bressan, R.A., Zhu, J.K., Bohnert, H.J.: Plant cellular and molecular responses to high salinity. - Annu. Rev. Plant Physiol. Plant mol. Biol. **51**: 463- 499, 2000.

Hoque, E.H., Mansfield, J.W.: Effect of genotype and explant age on callus induction and subsequent plant regeneration from root-derived callus of *Indica* rice genotypes. - Plant Cell Tissue Organ Cult. **78**: 217-223, 2004.

Jain, M., Choudhary, D., Kale, R.K., Bhalla-Sarin, N.: Salt- and glyphosate-induced increase in glyoxalase I activity in cell lines of groundnut (*Arachis hypogaea*). - Physiol. Plant. **114**: 499-505, 2002.

Johansen, K.S., Svendsen, II., Rasmussen, S.K.: Purification and cloning of the two domain glyoxalase I from wheat bran. - Plant Sci. **155**: 11-20, 2000.

Lafitte, H., Bennet, J.: Requirement for aerobic rice. Physiological and Molecular Considerations. - In: Bouman, B.A.M., Hengsdijk, H., Hardy, B. (ed.): Water-Wise Rice Production. Pp. 253. International Rice Research Institute, Los Baños 2003.

Maqbool, S.B., Husnain, T., Riazuddin, S., Masson, L., Christou, P.: Effective control of yellow stem borer and rice leaf folder in transgenic rice *Indica* varieties Basmati 370 and M7 using the novel delta-endotoxin cry2A *Bacillus thuringiensis* gene. - Mol. Breed. **4**: 501-507, 1998.

Mitsuoka, K., Honda, H., Xing, X.H., Unno, H.: Effect of intracellular 2,4-D concentration on plantlet regeneration of rice (*Oryza sativa* L.) callus. - Appl. Microbiol. Biotechnol. **42**: 364-366, 1994.

Murashige, T., Skoog, F.: A revised method for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-479, 1962.

Mutlu, S., Atici, O., Nalbantoglu, B.: Effects of salicylic acid and salinity on apoplastic antioxidant enzymes in two wheat cultivars differing in salt tolerance. - Biol. Plant. **53**: 334-338, 2009.

Oszvald, M., Kang, T.J., Tomoskozi, S., Jenes, B., Kim, T.G., Cha, Y.S., Tamas, L., Yang, M.S.: Expression of cholera toxin B subunit in transgenic rice endosperm. - Mol. Biotechnol. **40**: 261-268, 2008.

Paulus, C., Knollner, B., Jacobson, H.: Physiological and biochemical characterization of glyoxalase I, a general marker for cell proliferation, from a soybean cell suspension. - Planta **189**: 561-566, 1993.

Singh, A.K, Ansari, M.W., Pareek, A., Singla-Pareek, S.L.: Raising salinity tolerant rice: recent progress and future perspectives. - Physiol. mol. Biol. Plants **14**: 137-154, 2008.

Singla-Pareek, S.L., Reddy, M.K., Sopory, S.K.: Genetic engineering of the glyoxalase pathway in tobacco leads to enhanced salinity tolerance. - Proc. nat. Acad. Sci. USA **93**: 13404-13409, 2003.

Singla-Pareek, S.L., Yadav, S.K., Pareek, A., Reddy, M.K., Sopory, S.K.: Enhancing salt tolerance in a crop plant by overexpression of *glyoxalase II*. - Transgenic Res. **17**: 171-180, 2008.

Skipsey, M., Andrews, C.J., Townson, J.K., Jepson, I., Edwards, R.: Cloning and characterization of glyoxalase I from soybean. - Arch. Biochem. Biophys. **374**: 261-268, 2000.

Su, J., Hirji, R., Zhang, L., He, C., Selvaraj, G., Wu, R.: Evaluation of the stress-inducible production of choline oxidase in transgenic rice as a strategy for producing the stress-protectant glycine betaine. - J. exp. Bot. **57**: 1129-1135, 2006.

Tarczynski, M.C., Jensen, R.G., Bohnert H.J.: Expression of a bacterial *mtlD* gene in transgenic tobacco leads to production and accumulation of mannitol. - Proc. nat. Acad. Sci. USA **89**: 2600-2604, 1992.

Thach, T.N., Pant, R.C.: *In vitro* study on salt tolerance in rice. - Omonrice **7**: 80-88, 1999.

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

Yeo, A.R., Yeo, M.E., Flowers, S.A., and Flowers, T.J.: Screening of rice (*Oryza sativa* L.) genotypes for physiological characters contributing to salinity resistance, and their relationship to overall performance. - Theor. appl. Genet. **79**: 377-384, 1990.