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Improved performance of transgenic glycinebetaine-accumulating rice plants under drought stress

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Abstract

Plasmid DNA (pChlCOD), containing the selectable hygromycin phosphotransferase *hpt* gene for hygromycin B resistance and the *Arthrobacter globiformis codA* gene for choline oxidase which catalyzes the direct conversion of choline to glycinebetaine, was delivered into rice plants using *Agrobacterium*-mediated gene transfer *via* scutellum-derived calli. Southern, Northern and Western blot analyses demonstrated that the foreign gene had been transferred, integrated into rice chromosomal DNA and expressed. Drought test indicated that glycinebetaine acts as an osmoprotectant and its production in transgenic rice plant helped the cells to maintain osmotic potential and increased root growth, and thus enhanced the ability of the plants to tolerate water deficit

Additional key words: *Agrobacterium*-mediated transformation, choline oxidase gene, *Oryza sativa* L.

Introduction

Water stress induced by drought, salinity or freezing is the main limiting factor for plant growth and crop productivity. In response to these stresses, which all disturb the intercellular water balance, plants have evolved various protective mechanisms that allow them to acclimate to unfavorable environments for survival and growth. One such mechanism is the accumulation of certain organic metabolites of low molecular mass such as proline, glycinebetaine, trehalose, mannitol, and fructan (Sawahel and Hassan 2002, Tarczynski *et al.* 1992, 1993). These metabolites increase the ability of cells to retain water without disturbing normal cellular functions (Holmström *et al.* 1996).

Genetic engineering to increase content of some compatible solutes, such as proline, appears to be a promising approach to increase the ability of plants to tolerate environmental stress (Weretilnyk and Hanson 1990, Serrano and Gaxiola 1994, Bohnert *et al.* 1995). Recently, several different gene transfer approaches have been employed (Apse *et al.* 1999, Sakamoto and Murata 2000, Zhu 2000, Zhang and Blumwald 2001).

Several reports demonstrated that the metabolic engineering of plants leading to biosynthesis of glycinebetaine have enhanced tolerance to salt and cold stress (Saneoka *et al.* 1995, Hayashi *et al.* 1997, Sakamoto *et al.* 1998, Holmström *et al.* 2000). Here, we demonstrate that the accumulation of glycinebetaine through the genetic transformation of rice plant using the *codA* gene from *A. globiformis* have resulted a heritable improvement of drought tolerance.

Materials and methods

Plants and *Agrobacterium*-mediated gene transfer: Greenhouse-grown rice plants (*Oryza sativa* L. cv. G418) were used in transformation experiments. The plasmid pChlCOD (Fig. 1) (Sakamoto *et al.* 1998) was used for transformation of *Agrobaterium tumefaciens* EHA101. It contains the structural gene for hygromycin phospho-transferase *hpt* - which encodes resistance to the antibiotic hygromycin - and the *Arthrobacter globiformis*

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codA gene for choline oxidase that is terminated by the nopaline synthase *nos* gene terminator. Both genes are under the control of the cauliflower mosaic virus (CaMV) 35S promoter.

Seeds were surface sterilized in 70 % (v/v) ethanol for 2 min, followed by incubation in 4 % (m/v) $NaClO_3$ for 20 min and they were, then, washed four times in sterile water. Afterwards, they were germinated for 3 weeks on solid N6 medium (Chu 1978) containing 2 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) in order to induce callus from scutellum.

Plant genetic transformation was carried out according to Hiei *et al.* (1994). 3-week old scutellum-derived calli were co-cultured with an overnight culture of *A. tumefaciens* strain EHA101 harboring pChlCOD in the presence of 150 μM acetosyringone. After 3 d, infected calli were placed on N6 medium containing 50 mg dm^{-3} hygromycin B as a selective agent, 250 mg dm^{-3} cefotaxime and 2 mg dm^{-3} 2,4-D. After 3 weeks, surviving calli were transferred to regeneration medium and allowed to form shoots and roots in the presence of hygromycin B and cefotaxime (Sawahel 1996, Sakamoto *et al.* 1998). Plantlets with roots of about 1 cm length were potted into soil and transferred to the greenhouse.

Southern blot analysis: Total DNA was isolated from transgenic and control rice plants according to Dellaporta *et al.* (1983). Thereafter, it was digested with *Hin*dIII, electrophoresed through 1.0 % (m/v) agarose gel and transferred to nylon membranes (Southern 1975). The 0.2 kb fragment that contains *codA* gene for choline oxidase was released from pChlCOD plasmid by *Nde*I/*Sac*I digestion. Radioactive probe was made with ^{32}P using random-primed labeling (Feinberg and Vogelstein 1983). The prehybridization blots were hybridised with *codA* probe overnight at 65 °C, and washed three times with sodium phosphate buffer (pH 7.2) and 1.0 % (m/v) sodium dodecyl sulfate (SDS) at 65 °C for 5, 30, and 15 min, and visualized by auto-radiography.

Northern, and Western blot analyses: Total RNA, 15 μg, isolated from transgenic and control rice plants (Chomczynski and Sacchi 1987) was subjected to electrophoresis in a 1.2 % (m/v) agarose gel, transferred to the GeneScreen membrane, and hybridised to *codA probe* labeled with [^{32}P]dATP. Hybridisation was carried out at 50 °C, and filters were exposed to X-ray film at -70 °C (Church and Gilbert 1984).

For Western blot analysis, 1 g of fresh leaves from transgenic and control rice plants were homogenized in 5 cm^{-3} of the extraction buffer (20 mM sodium phosphate, pH 7). Ten μg of protein was separated by 8 % SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane (*Schleicher & Schuell*, Dassel, Germany), and were allowed to react with an antiserum raised against choline oxidase (Deshnium *et al.* 1995).

Determination of glycinebetaine content: In order to examine the accumulation of glycinebetaine in transgenic rice plants, quaternary ammonium compounds were extracted from leaves and analyzed by proton-NMR spectrometry as described by Hayashi *et al.* (1997).

Drought tolerance tests: Transgenic plants were grown in 9-cm pots filled with a 1:1 mixture of *Perlite* and *Vermiculite*. They were grown under continuous irradiance of approximately 25 μmol m^{-2} s^{-1} at temperature of 22 °C. Three-week-old plants were exposed to drought stress induced by withholding water for 2 weeks (Kasuga *et al.* 1999). The plants were then transferred to pots under normal growing conditions for 3 weeks. The numbers of plants that survived and continued to grow were counted.

Leaf sap was squeezed through a syringe from freshly picked leaves of transgenic and control rice plants. The osmotic potentials of expressed leaf sap were measured according to Kishor *et al.* (1995) using a vapour pressure osmometer (model *5100 C, Wescor;* Logan, UT, USA). Potassium chloride solutions were used as standards at 25 °C and the chamber was equilibrated for different time periods (1 - 3 min) before taking measurements.

Evaluation of the growth performance under water-deficit condition was carried out using 3-week-old seedlings that had three leaves, and some seedlings had an emerging fourth leaf. These plants were grown in soil in small pots with holes in the bottom. The pots were kept in flat-bottom trays containing water. Before and after stress treatments, the initial plant height, leaf number, and leaf length for transgenic and control rice plants were measured. The mean value of the 7 tested plants in each treatment was calculated and used for comparing the transgenic plants with the control plants.

Results

Generation of transgenic rice plants: *Oryza sativa* was transformed with *codA* gene carried by the plasmid DNA (pChlCOD) (Fig. 1). From 200 calli, we obtained 15 transgenic plants that were resistant to hygromycin B. Visual examination of transgenic plants indicated no apparent phenotypic differences as compared with control plants during regeneration in axenic culture.

Southern blot analysis (Fig. 2*A*) was carried out using genomic DNA isolated from a transgenic line of primary transformant plants R_0. The probe for *codA* gene was hybridised to genomic DNA of the transgenic line, which had been digested with *Hin*dIII, and a hybridisation signal

of expected size of about 2.0 kb was detected (Fig. 2*A*, *lane c*). The probe was further hybridised to undigested DNA of the transgenic line. Hybridisation occurred only to the uncut high molecular mass chromosomal plant DNA, and not to smaller pieces of DNA, indicating that the expressed transgene had integrated into chromosomal DNA of the transgenic plants (Fig. 2*A*, *lane b*). There was no hybridisation signal from the untransformed rice DNA (Fig. 2*A*, *lane a*).

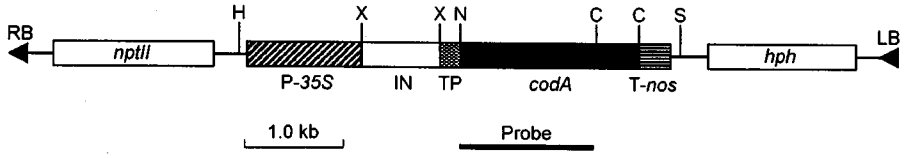

Fig. 1. Schematic representation of the T-DNA region of the plasmid pChICOD used for producing transgenic rice plants. *CodA* - gene encoding choline oxidase, *hpt* - gene conferring resistance to hygromycin, *nptII* - gene conferring resistance to kanamycin, P-*35S* - 35S RNA promoter from cauliflower mosaic virus, IN - first intron of the *SodCc2* gene from rice, T-*nos* - polyadenylation signal from a gene for nopaline synthase, TP - sequence from the *RbcS* gene from rice that encodes the transit peptide, LB - left border, RB - right border. Abbreviations for restriction sites: C - *Sac*I, H - *Hin*dIII, N - *Nde*I, S - *Sal*I, X - *Xba*I. The *horizontal bar* refers to the *codA*-coding region for the synthesis of the probe.

Expression of the *codA* gene in transgenic rice plants: In order to select elite candidates for drought test, the expression of *codA* was examined in transgenic rice plants. The *codA* transcript in transgenic rice plants was determined by Northern blot analysis (Fig. 2*B*). Signals that were slightly longer than the expected length (2.0 kb) were detected in the leaves of transgenic plants. This is most likely because of the inclusion of sequence of 0.2 kb in the used plasmid that encoded the transit peptide (Sakamoto *et al.* 1998). There was no signal from the untransformed control.

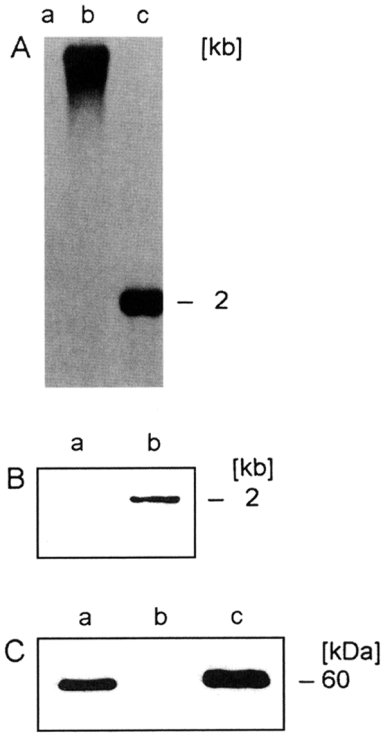

Fig. 2. Molecular analysis of transgenic rice plants. *A* - Southern blot analysis of genomic DNA from rice plants: *lane a* - DNA isolated from an untransformed plant, *lanes b, c* - uncut and *Hin*dIII-digested DNA from a transformed plant. *B* - Northern blot analysis of total RNA from rice plants: *lane a* - RNA from an untransformed plant, *lane b* - RNA from a transgenic plant. *C* - Western blot analysis of total soluble proteins from rice plants: *lane a* - authentic choline oxidase, *lane b* - protein from an untransformed plant, *lane c* - protein from a transgenic plant.

Leaf extracts prepared from the selected transformants were subjected to Western blot analysis with antiserum raised against choline oxidase. A specific positive band of approximately 60 kDa, which was consistent with the molecular mass of standard choline oxidase, was clearly detected in transgenic plants but not in the non-transgenic control plants (Fig. 2*C*).

Glycinebetaine detection in transgenic rice plants: The control rice plants couldn't produce glycinebetaine as a consequence of the apparent absence of the two enzymes required for its biosynthesis (Flowers *et al.* 2000). However, transgenic rice plants accumulated 1.3 ± 0.3 μmol g^{-1}(f.m.) glycinebetaine in leaves and no significant difference in the content of choline between transgenic (0.6 ± 0.2 μmol g^{-1}) and non-transgenic (0.5 ± 0.1 μmol g^{-1}) rice plants was found. This indicated that choline is not rate-limiting for the synthesis of glycinebetaine in transgenic plants (Sakamoto *et al.* 1998).

Drought tolerance of transgenic plants: To test whether the expression of *codA* gene, which catalyzes the direct conversion of choline to glycinebetaine, enhanced tolerance to drought in transgenic rice plants, the following tests have been carried out:

In dehydration test 3-week-old seedlings of control and transgenic rice plants were not watered for 2 weeks and only 1 out of 110 (0.9 %) control plants survived this 2-week period, whereas 92 out of 130 (70.8 %) transgenic glycinebetaine-accumulating rice plants survived and

continued to grow after rewatering. The dehydration effect was more pronounced in lower leaves of the plants, which are more sensitive to water stress and start wilting first during the drought stress on the plants.

To get more insight about the stress situation and the reactions of transgenic and control rice plants, the osmotic potentials in the leaf cells were measured before and after water stress (Table 1). Before water stress, small differences in the osmotic potentials of the leaf sap were observed between transgenic and control rice plants. During water stress, the osmotic potentials in control plants declined from about -0.71 to -1.04 MPa in the top leaves and from -0.72 to -1.23 MPa in the lower leaves. In transgenic rice plants, however, smaller changes of osmotic potentials were observed. The osmotic potentials of leaf sap from transgenic plants decreased under water-stress from about -0.70 to -0.71 MPa in the top leaves and only to about -0.73 in the lower leaves.

Table 1. Osmotic potential [MPa] of rice leaf sap from control and transgenic rice plants before and after water stress. Means ± SE of 5 independent experiments.

		Top leaf	Middle leaf	Lower leaf
Before stress	control	-0.71 ± 0.02	-0.67 ± 0.04	-0.72 ± 0.02
	transgenic	-0.70 ± 0.04	-0.74 ± 0.16	-0.69 ± 0.19
After stress	control	-1.04 ± 0.03	-1.08 ± 0.05	-1.23 ± 0.04
	transgenic	-0.71 ± 0.13	-0.72 ± 0.17	-0.73 ± 0.14

Glycinebetaine content in leaves of 5 transgenic plants under water stress was 4.1 ± 0.3 μmol g^{-1} (f.m.) compared to 1.5 ± 0.2 μmol g^{-1} (f.m.) before water stress. No glycinebetaine was detected in control rice plants before and after water stress. These results are comparable with the observed changes in osmotic potential of transgenic proline-accumulating tobacco plants under water stress (Kishor *et al.* 1995) suggesting that glycinebetaine accumulation in transgenic plants has a similar effect to the osmoprotectant proline in helping the cells to maintain pressure potential and thus enhancing the ability of the plants to tolerate water stress.

A significant difference between the growth performance of transgenic and control rice plants was observed under water deficit induced by withholding water from the tray. Leaves of both control and transgenic plants were wilted after 6 d, but wilting of transgenic plants leaves started 3 d later than the control and was less severe. The growth of the young leaves of both control and transgenic plants were inhibited, but transgenic plants maintained higher growth rates than control plants.

Data collected 28 d after the beginning of initial water deficit (4 cycles of a 5 d water deficit followed by a 2 d recovery) showed that the average plant height and root fresh mass were significantly higher in transgenic rice plants (Table 2). This result is comparable with the observed growth performance of transgenic fructan- and HVA1-accumulating plants under water stress (Pilon-Smits *et al.* 1995, Xu *et al.* 1996) indicating that enhanced root development seems to be an adaptation to drought and a possible basis for better growth performance of the whole plant. This may lead to the speculation that glycinebetaine might effect root formation similarly as mannitol (Tarczynski *et al.* 1993).

Table 2. Growth performance of transgenic rice plants under water deficit. The growth rate was calculated as the percentage length increase of the two upper leaves during the 3-day period of water stress. The plant height was calculated as the mean length of the two longest leaves at the top of the plants. Means of 7 plants ± SE.

	Leaf growth [%]	Plant height [cm]	Root f.m. [g]
Control	58	18 ± 1.9	0.7 ± 0.2
Transgenic	143	39 ± 1.1	2.9 ± 0.1

Inheritance of the *codA* gene and drought tolerance in the R_1 generation: In order to investigate the inheritance of drought tolerance, 16 primary transgenic R_0 rice plants were self-pollinated and the 37 R_1 seeds obtained were screened for hygromycin B resistance, examined for the presence, and expression of *A. globiformis codA* gene by Southern, Northern and Western blot analyses and tested for drought tolerance.

It was found that 25 of 37 R_1 plants exhibited tolerance to water-deficit conditions. These progenies were found to harbor the *codA* gene as exactly shown in the R_0 plants (Fig. 2*A*). The segregation of drought tolerance among progenies was in accordance with the predicted Mendelian ratio of 3:1 (tolerant:sensitive), as tested by χ^2 analysis ($\chi^2 = 0.078$, $P > 1$ %).

Discussion

Previous reports indicated that the genetic engineering of the synthesis of osmoprotectants such as fructan, mannitol, trehalose or proline in higher plants (Pilon-Smits *et al.* 1995, Tarczynski *et al.* 1993, Holmström *et al.* 1996, Kishor *et al.* 1995) increased tolerance to the water deficit. In this study, we demonstrate the potential usefulness of glycinebetaine (*N,N,N*-trimethylglycine) in the improvement of drought tolerance of rice plants.

Metabolic engineering is an approach by which cellular activities can be altered through the use of

recombinant DNA technology. The application of this approach for the biosynthesis of glycinebetaine using the *codA* gene has been the focus of considerable attention as a potential strategy for the enhancement of tolerance to cold and salt stresses in sensitive species such as the cyanobacterium *Synecochoccus* sp. and *Arabidopsis thaliana* (Hayashi *et al.* 1997, Deshnium *et al.* 1995).

The biosynthetic pathways for glycinebetaine have been characterised in higher plants and in microorganism, such as *Escherichia coli* and the soil bacterium *Arthrobacter globiformis* (Weretilnyk and Hanson 1990). In both higher plants and *E. coli,* two enzymes are required for the production of glycinebetaine *via* the two-step oxidation of choline, with glycinebetaine aldehyde as the unstable intermediate. By contrast, in *A. globiformis,* only a single enzyme is required, namely choline oxidase, which catalyzes the direct conversation of choline to glycinebetaine.

Glycinebetaine is regarded as an extremely effective compatible solute that accumulates in the chloroplasts of certain plants, such as spinach and barley, when these plants are exposed to environmental stresses (Sakamoto and Murata 2000). Under stress conditions, glycinebetaine not only allows cells to adjust the osmotic potential in their cytoplasm to maintain an appropriate water content, but it protects proteins from the stress-induced dissociation of their respective subunits (Egan *et al.* 2001). In photosynthetic organisms, glycinebeatine has profound effects on the ability of the oxygen-evolving photosystem 2 complex at high concentration of NaCl. A major role of glycinebetaine might be to protect membranes and macromolecules from damaging effect of stress. It also possible that glycinebetaine might be compartmentalized within cells such that, at certain sites, the concentration of glycinebetaine might be enough to confer substantial protection against stress even when the overall accumulation is low. Moreover, recent studies have provided new insights into the possible roles of glycinebetaine in cell under stress (Flowers *et al.* 2000).

In conclusion, as many agronomically important crops, such as potato, are glycinebetaine-deficient, genetic engineering of glycinebetaine from choline into other plant species might provide a possible and reliable strategy for the production of stress tolerant plants.

References

Apse, M., Aharoni, G., Snedden, W., Blumwald, E.: Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in *Arabidopsis*. - Science **285**:1256-1258, 1999.

Bohnert, H., Nelson, D., Jensen, R.: Adaptations to environmental stresses. - Plant Cell **7**: 1099-1111, 1995.

Chomczynski, P., Sacchi, N.: Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. - Anal. Biochem. **162**: 156-159, 1987.

Chu, C.: The N6 medium and its applications to another culture of cereal crops. - In: Yewam, E. (ed.): Proceedings of the Symposium on Plant Tissue Culture. Pp. 43-50. Science Press, Beijing 1978.

Church, G., Gilbert, W.: Genomic sequencing. - Proc. nat. Acad. Sci. USA **81**: 1991-1995, 1984.

Dellaporta, S., Wood, J., Hicks, J.: A plant DNA minipreparation: Version II. - Plant mol. Biol. Rep. **1**: 19-21, 1983.

Deshnium, P., Los, D., Hayashi, H., Mustardy, L., Murata, N.: Transformation of *Synechococcus* with a gene for choline oxidase enhances tolerance to salt stress. - Plant mol. Biol. **29**: 897-907, 1995.

Egan, T., Dewald, H., Ungar, I.: The effect of different salts of sodium and potassium on the accumulation of glycinebetaine in *Atriplex prostrata*. - Biol. Plant. **44**: 595-597, 2001.

Feinberg, A., Vogelstein, B.: A technique for radio-labelling DNA restriction endonuclease fragments to high specific activity. - Anal. Biochem. **132**: 6-13, 1983.

Flowers, T., Koyama, M., Flowers, S., Sudhakar, C., Singh, K., Yeo, A.: QTL: their place in engineering tolerance of rice to salinity. - J. exp. Bot. **51**: 99-106, 2000.

Hayashi, H., Alia, Mustrady, L., Deshnium, P., Ida, M., Murata, N.: Transformation of *Arabidopsis thaliana* with the *codA* gene for choline oxidase; accumulation of glycinebetaine and enhanced tolerance to salt and cold stress. - Plant J. **12**: 133-142, 1997.

Hiei, Y., Ohta, S., Komari, T., Kumashiro, T.: Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. - Plant J. **6**: 271-282, 1994.

Holmström, K., Somersalo, S., Mandal, A., Palva, T., Welin, B.: Improved tolerance to salinity and low temperature in transgenic tobacco producing glycinebetaine. - J. exp. Bot. **51**: 177-185, 2000.

Holmström, K., Welin, E., Palva, A., Tunnela, O., Londesborough, J.: Drought tolerance in tobacco. - Nature **379**: 683-684, 1996.

Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K., Shinozaki, K.: Improving plant drought, salt and freezing tolerance by gene transfer of a single stress-inducible transcription factor. - Natur. Biotechnol. **17**: 287-291, 1999.

Kishor, B., Hong, Z., Miao, G., Hu, C., Verma, D.: Overexpression of Δ^1-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. - Plant Physiol. **108**: 1387-1394, 1995.

Pilon-Smits, E., Ebskamp, M., Paul, M., Jeuken, M., Weisbeek, P., Smeekens, S.: Improved performance of transgenic fructan-accumulating tobacco under drought stress. - Plant Physiol. **107**: 125-130, 1995.

Sakamoto, A., Murata, N.: Genetic engineering of glycinebetaine synthesis in plants: current status and implications for enhancement of stress tolerance. - J. exp. Bot. **51**: 81-88, 2000.

Sakamoto A., Alia, Murata, N.: Metabolic engineering of rice leading to biosynthesis of glycinebetaine and tolerance to salt and cold. - Plant mol. Biol. **38**: 1011-1019, 1998.

Saneoka, H., Nagasaka, C., Hahn, D., Yang, W., Premachandra, G., Joly, R., Rhodes, D.: Salt tolerance of glycinebetaine-

deficient and -containing maize lines. - Plant Physiol. **107**: 631-638, 1995.

Sawahel, W.: Identification and molecular analysis of rice somatic hybrids using foreign transgenes. - Biotechnol. Lett. **18**: 909-912, 1996.

Sawahel, W., Hassan, A.: Generation of transgenic wheat plants producing high levels of the osmoprotectant proline. Biotechnol. Lett. **24**: 721-725, 2002.

Serrano, R., Gaxiola, R.: Microbial models and salt stress tolerance in plants. - Crit. Rev. Plant Sci. **13**: 121-138, 1994.

Southern, E.: Detection of specific sequences among DNA fragments separated by gel electrophoresis. - J. mol. Biol. **98**: 503-517, 1975.

Tarczynski, M., Jensen, R., Bohnert, H.: Stress protection of transgenic tobacco by production of the osmolyte mannitol. - Science **259**: 508-510, 1993.

Tarczynski, M., Jensen, R., Bohnert, H.: 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.

Weretilnyk, E., Hanson, A.: Molecular cloning of a plant betaine-aldehyde dehydrogenase, an enzyme implicated in adaptation to salinity and drought. - Proc. nat. Acad. Sci. USA **87**: 2745-2749, 1990.

Xu, D., Duan, X., Baiyang, W., Hong, B., Ho, T., Wu, R.: Expression of a late embryogenesis abundant protein gene, *HVA1*, from barley confers tolerance to water deficit and salt stress in transgenic rice. - Plant Physiol. **110**: 249-257, 1996.

Zhang, H., Blumwald, E.: Transgenic salt-tolerance tomato plants accumulate salt in foliage but not in fruit. - Natur. Biotechnol. **19**: 765-768, 2001.

Zhu, J.: Genetic analysis of plant salt tolerance using *Arabidopsis*. - Plant Physiol. **124**: 941-948, 2000.