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OVER-EXPRESSION OF THE *CODA* GENE BY *RD29A* PROMOTER IMPROVES SALT TOLERANCE IN *NICOTIANA TABACUM*

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Abstract

Soil-salinity is a serious problem that impedes food production and reforestation worldwide. In order to improve plant salt tolerance, many genes for abiotic stress have been over-expressed by genetic engineering. The *codA* gene encodes for the choline oxidase of the soil bacterium *Arthrobacter globiformisa*. We analyzed the effect of overexpression of *CodA* on stress tolerance in transgenic tobacco plants using the stress-inducible *Rd29A* promoter. Four transgenic *Nicotiana tabacum* plants were obtained. Southern blot analysis demonstrated that the *codA* gene had integrated into the genome of the tobacco. When exposed to NaCl salt stress, the chlorophyll content of the transgenic plants was not altered significantly. With prolonged stress (2-4 weeks), superoxide dismutase (SOD) activity increased by at least 50% relative to controls in all four transgenic lines. Malondialdehyde (MDA) accumulation was observed after 7 days of NaCl stress in all transgenic plants, but decreased during continued salt stress in some lines, indicative of salt stress tolerance. These results showed that the *codA* gene may enhance salt-tolerance by inhibiting lipid peroxidation through induction of stress response genes like SOD. These results offer additional support to the suggestion that *CodA* is involved in the modulation of plant gene activity in response to salt stress conditions and indicate that plants over-expressing *codA* could be engineered for agriculture in saline soils.

Introduction

The development and survival of plants is constantly challenged by abiotic stresses, such as drought, salinity, extreme temperatures, chemical toxicity and oxidative stress (Bray *et al*., 2000). Salinity is a major abiotic stress factor affecting plants growth and productivity world-wide (Ahmad *et al*., 2012). Plants elicit complex physiological and molecular responses to tide over these adversities. Stress is perceived and transduced through a chain of signaling molecules that ultimately affect regulatory elements of stress-inducible genes to initiate the synthesis of different classes of proteins or alter their activities (Mukhopadhyay *et al*., 2004). Stress-regulated genes are the controlling basis of stress response in plants. Therefore, the biological roles of the gene products are studied directly or deduced from sequence homologies to previously studied proteins of both plant and non-plant origin. Up-regulated salt stress operates in almost all aspects of plant function and metabolism including signal transduction, ion homeostasis, carbohydrate and nitrogen metabolism, photosynthesis, growth and development (Kalifa *et al*., 2004).

Several different genes transfer approaches have been employed to improve the salt tolerance of plants (Holmberg, N. *et al*., 1998). Gene transformation has played an important role in plant improvement and can shorten the breeding period or breed varieties with high-salt tolerance. Transferred genes included those encoding enzymes required for the biosynthesis of various osmoprotectants (Hayashi *et al*., 1993; Kavi, *et al*., 1995; Tarczynski *et al*., 1997), genes encoding enzymes that modify membrane lipids (Kodama *et al*., 1994; Ishizaki-Nishizawa, *et al*., 1996), genes for the late embryogenesis abundant (LEA) proteins (Xu, *et al*., 1996), and genes for various detoxification enzymes (McKersie, 1996). A significant number (nearly half) of the salt-stress-regulated genes encode proteins whose roles cannot be predicted from their primary amino acid sequences (Aubourg & Rouze 2001; Bohnert *et al*., 2001). Thus, the biological activity of the majority of these proteins remains to be determined (Ingram & Bartels 1996; Bray 1997; Hasegawa *et al*., 2000), and deciphering the pathways in which stress proteins operate will assist in development of transgenic plants with improved salinity tolerance.

Choline oxidase from the soil bacterium *Arthrobacter globiformisa* that catalyzes the conversion of choline into betaine (Ikuta *et al*., 1977). Betaine protects cells from salt stress by maintaining an osmotic balance with the environment (Robinson & Jones, 1986) and by stabilizing the quaternary structure of complex proteins (Bernard *et al*., 1988; Santoro *et al*., 1992; Papageorgiou & Murata, 1995). In photosynthetic systems, betaine stabilizes the oxygen-evolving photosystem II complex (Murata *et al*., 1992; Papageorgiou & Murata, 1995; Papageorgiou *et al*., 1991) and ribulose bisphosphate carboxylase (Rubisco) at high concentrations of NaCl (Incharoenskdi *et al*., 1986). Hayashi (1997) reported that over-expression of *codA* allowed transgenic *Arabidopsis thaliana* to grow well in 100 mM NaCl while wild type plants grown under similar conditions displayed a reduction in leaf size and chlorosis. He *et al*., (2001) over-expressed *codA* in tobacco plants and reported growth at 300mM NaCl while wild type plants died under similar conditions. Hitesh *et al*., (2009) over-expressed *codA* gene in the indica rice plant and

produced transgenic plants during seedling, vegetative, and reproductive stages. Transgenic plants maintained higher photosystem II activity and exhibited better physiological tolerance to water stress than wild type plants. Thus, increased osmotic balance by enhanced betaine synthesis confers significant salt tolerance in a variety of plant species. Unfortunately, most transgenic plants using the targeted genes under the control of the CaMV 35S promoter exhibit morphological abnormalities, restricting the potential for use in commercial production. This occurs because overproduction of targeted products during plant growth interferes with normal development (Gan & Amasino, 1997). To achieve better control of gene overproduction, researchers fused it to inducible promoters that were activated by stress.

Nicotiana tabacum is an important model plant for transgenesis research. Use of the stress-inducible *Rd29A* promoter instead of the constitutive 35S CaMV promoter for the overexpression of *CodA* minimizes the negative effects on plant growth. In order to further clarify the biological role of *CodA in vivo*, we constructed a plasmid containing the abiotic stress-inducible promoter *Rd29A* to control *codA* gene expression, and transformed *Nicotiana tabacum* by Agrobacterium-mediated gene transfer. The results of our analysis indicated that transgenic plants over-expressing the *codA* gene demonstrate dramatically increased salt tolerance, growing normally in the presence of 150mM NaCl. The results of the present study will offer additional support to the suggestion that *CodA* is involved in the modulation of plant gene activity in response to salt stress conditions. Plants over-expressing *codA* could be engineered for agriculture in saline soils.

Materials and Methods

Construction of binary vector: The plasmid *pGAH/codA* containing the *codA* gene was kindly provided by Norio Murata (Department of Regulation Biology, National Institute for Basic Biology). The vector pCAMBIA1300 was generated in our laboratory. The *pGAH/codA* plasmid was digested with *Xba* I and *Kpn* I (Takara Dalian, China), and the smaller fragment (2.5 kb) was cloned into pUC19Rd-nos containing the inducible promoter *Rd29A*. This new recombinant plasmid was digested with *Hind* III and *Kpn* I and the smaller fragment (3.4 kb) was cloned into pCAMBIA1305. The resulting plasmid was named pBS1305*Rd29codA* (Fig. 1) and was introduced into *A. tumefaciens* EHA105 (Hood *et al*., 1993) using the freeze-thaw method (Holsters *et al*., 1978).

Agrobacterium-mediated transformation: Agrobacterium-mediated leaf disc transformation of tobacco was carried out per standard protocol (Murashinge & Skoog 1962). *Agrobacterium* containing the vector pBS1300*Rd29codA* was inoculated into YM medium supplemented with 50 mg L^{-1} kanamycin and 25 mg L^{-1} rifampicin and grown at 28°C for 3 days. Leaf discs of 6 mm diameter were transferred into a sterile flask containing the Agrobacterium suspension (OD_{600}=0.3-0.6) and soaked for 30 min with occasional shaking. The discs were then dried on a pad of sterile filter paper to remove excess surface water. The explants were plated onto shoot induction medium composed of MS medium supplemented with 1 mg L^{-1} 6-benzylaminopurine (6-BA), 6g L^{-1} agar, 30g L^{-1} sucrose and 50mg L^{-1} acetosyringone (AS) (pH 5.8). Explants were cultured in the dark at 25±1°C for 3 days and then transferred onto the selection medium composed MS supplemented with 1 mg L^{-1} 6-BA, 30 g L^{-1} sucrose, 300 mg L^{-1} cefotaxime, 7 g L^{-1} agar (pH 5.6-5.8), and cultured under a 16/8-h (light/dark) photoperiod at 25 ± 1°C. Regenerated shoots were separated from the explants and transferred to 1/2 MS medium with 0.3mg L^{-1} IBA, 30g L^{-1} sucrose and 300mg L^{-1} cefotaxime for rooting. The well-developed plantlets were then transferred to a greenhouse (Fig. 2). The integration and expression of *CodA* was ascertained by Southern blot analysis asfallow:

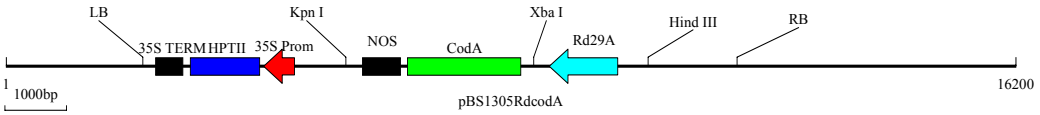

Fig. 1. Diagrammatic representation of the binary plasmid vector pBS1305RdcodA. The plasmid vector contains the following elements.

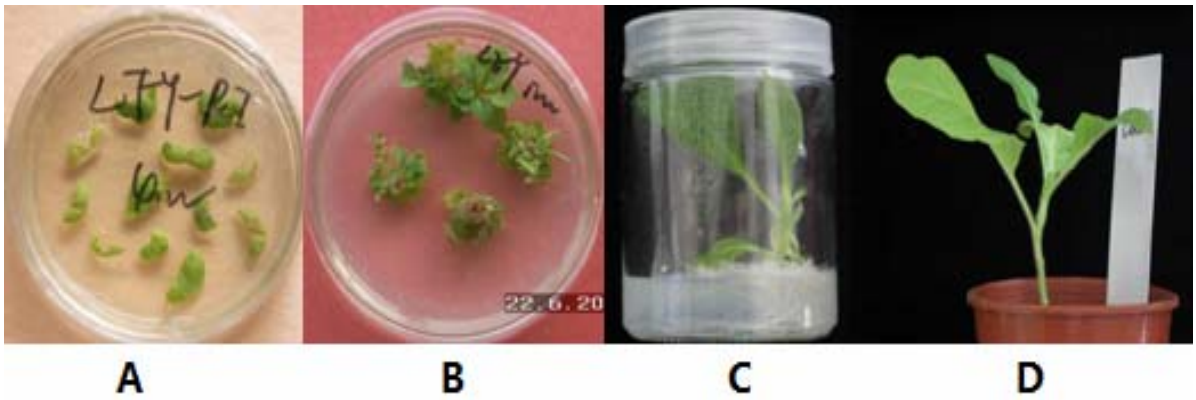

Fig. 2. *Nicotiana tabacum* transformation and plant regeneration; (A) Leaf disks (B) Shoot induction from leaf disks (C) Rooting (D) Transplanting

PCR analysis: Genomic DNA was extracted from the leaves of transformed and non-transformed plants according to the CTAB (cetyltri methyl ammonium bromide) method described by Doyle & Dickson (1987). Standard PCR was carried out in a 25µL reaction mixture containing 100ng DNA, 250µM of each dNTP, 10 pmol of each primer, 1.25 U Taq DNA polymerase, 2mM $MgCl_2$, and the 1×Taq buffer. The putative transgenic clones were PCR-tested for the *Rd29A* and *codA* genes. The following primers were used in the PCR. The *Rd29A* PCR reaction was performed in a PE9600 PCR thermal cycler using an initial denaturation of 94°C for 4 min., followed by 30 cycles of 94°C for 45 s, 48°C for 30s, 72°C for 60 s, and then a final elongation step at 72°C for 10 min. Conditions for *codA* detection were 94°C for 4 min. and 30 cycles of 45 s at 94°C, 45 s at 63°C, and 60 s at 72°C, followed by an extension at 72°C for 10 min. The amplified products were visualized on a 1.5% agarose gel.

Southern blotting: Southern blotting was performed according to standard protocols (Sambrook *et al*., 1989). Briefly, genomic DNA was isolated from the leaves of transgenic plants according to the method described by Lu & Zheng (1992). Fifty micrograms of DNA was digested with *Hind* III overnight at 37°C. The digested products were separated on a 1.2% agarose gel and transferred to a Hybond N^+ nylon membrane (Amersham Biosciences, Roosendaal, Netherlands). Hybridization and detection were carried out using ECL direct labeling and detection systems according to the directions from the manufacturer (GE Healthcare, USA). The ECL-labeled probes were amplified from the Pbs1305*RdcodA* plasmid using the *codA* primers. The PCR products were purified from a 1.2% agarose gel using the UNIQ-10 Column Gel Extraction Kit (Sangon, Shanghai, China).

Semi-quantitative RT-PCR: Five-month-old transgenic plants and control plants were treated by Hoagland's liquid medium contain 200 mM NaCl for 4 days. Total RNA was extracted from the leaves of treated plants by Trizol solution (Invitrogen, USA). Reverse transcription (RT)-PCR, using the Access RT-PCR System kit (Promega, USA), was carried out to confirm the transgene expression. From each RT-PCR sample, 1µg of total RNA was used in a 10µL RT reaction using oligo dT as a primer. For amplification of *codA*, 2µL of the cDNA was used for amplification in a 20µL PCR reaction using primers corresponding to the C-terminus of *codA* (F 5'-AATCGGGCTACGACTGG-3' and R 5'-GCGTCCTCGTTGGTTTC-3'). Thirty PCR cycles (95˚C for 20 s, 55˚C for 1 min and 72˚C for 1 min) were performed using PE9600 (PE Co., USA).

Stress treatments and physiological studies: The transgenic plants were soaked in Hoagland's liquid medium containing 0, 50, 100, or 150 mM NaCl. After every 7 days, leaf tissue (2g) was ground in liquid nitrogen and homogenized in 10 volumes of extraction buffer (10 mM Sodium phosphate and 1% Sodium metabisulphite, pH 7.0) per unit fresh weight. After centrifugation at 12000×*g* for 30 min at 4˚C, total SOD activity was assayed by the method described in Giannopolitis & Rise (1977). Malondialdehyde (MDA), a biomarker for lipid peroxidation, was assayed by the method described in Peever & Higgins (1989). Chlorophyll content was detected by the method described in Arnon (1949).

Statistical analysis: Data analyses were conducted using SPSS version 16.0 statistical software (SPSS Inc., Chicago, Ill.). For all analyses, the level of significance was set at $p<0.05$. Sample variability is given as the standard deviation (S.D.).

Results

When plant cells were cultured in a medium containing kanamycin, the cells that expressed the NPT gene were able to survive. Non-transformed *Nicotiana tabacum* leaves (with no kanamycin activity) did not grow in media with 300 mg L^{-1} kanamycin or more. After transformation, a number of shoots could be induced in the presence of 300 mg L^{-1} kanamycin. Following 2 months of culture in the selective medium, regenerated buds (Figs. 2A and B) were isolated and transferred to a rooting medium (Figs. 2C and D). The resulting plants were considered to be (putative) independent transgenic plant lines. The well-developed plantlets were then transferred to a greenhouse (Fig. 2E). Twenty independent transgenic plantlets were successfully grown.

Stress-induced expression of codA gene: Following *A. tumefaciens*-mediated transformation, twenty independent putative transgenic plantlets were assayed by PCR for the presence of the *Rd29A* and *codA* genes. Analysis of the proliferating plantlets revealed that just four plants were positive for both the *Rd29A* gene (PCR product of about 891 bp) (Fig. 3) and the *codA* gene (PCR product of about 825 bp) (Fig. 4).

Gene integration analysis was then performed by Southern blotting (Fig. 5). Genomic DNA from the putative transgenic lines was digested by *Hind* III, which has a unique restriction site located in the T-DNA region (Fig. 1). Southern blot analysis demonstrated that all *Rd29/codA* positive plants were transgenic lines with a single insert. No bands were observed in blots from wild type plant.

RT-PCR: Expression profile of the *codA* gene in 4 transgenic plants was monitored by RT-PCR and it was revealed that the *codA* gene was expressed normally in transgenic lines, while no detectable expression of *codA* was observed in wild type plants (Fig. 6).

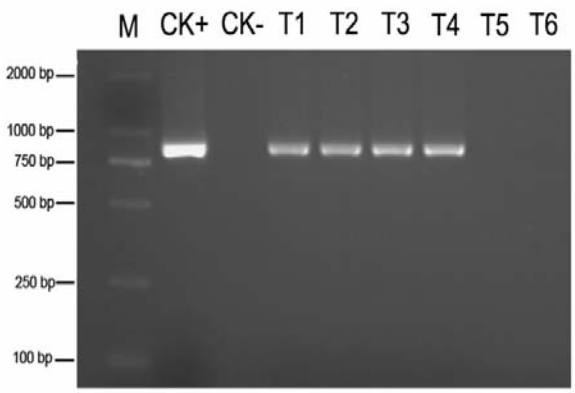

Fig. 3. Detection of *Rd29A* by PCR
Lanes: DL2000, DNA marker (Takara, China), CK+:plasmid *pBS1305RdcodA* , Ck-: wild type plant, T1-T4:positive plantlets, T5,T6:negative plants.

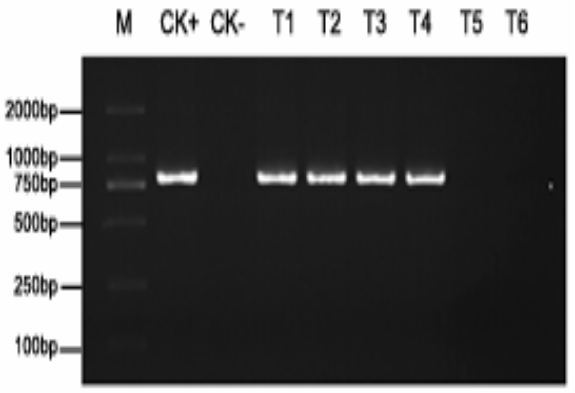

Fig. 4. Detection of *codA* by PCR
Lanes: DL2000, DNA marker (Takara, China),CK+: plasmid *pBS1305RdcodA*, Ck-: wild type plant,T1-T4: positive plantlets,T5,T6: negative plants.

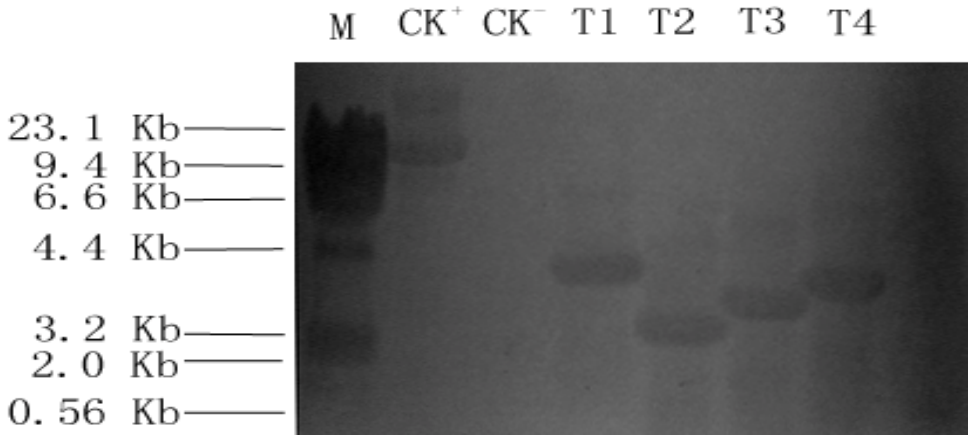

Fig. 5. Southern blot analysis of transformed plantlets.
DNA was digested with *Hind* III and probed with an ECL-labeled fragment containing the *codA* gene from the pBS1305RdcodA plasmid. Lane 1 plasmid; Line 2 wild type plant Lanes 3-6 transgenic plants; ; M molecular marker.

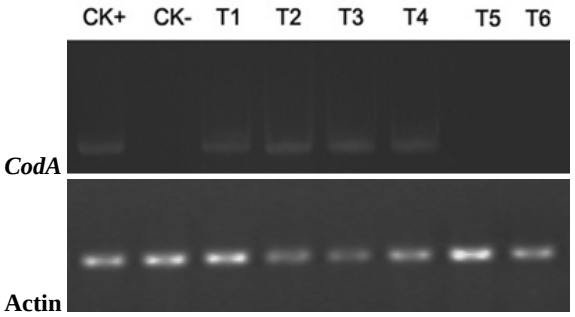

Fig. 6. RT-PCR analysis of *codA* gene expression
Lanes: M, DNA marker (DL2000), 1-4 transgenic plants (T1; T2; T3; T4), T5; T6 wild type plant

Analysis of salt tolerance

Physiological studies of transgenic plants under stress: During culture in the greenhouse, the 4 transgenic plants (T1-4) were assayed for salt tolerance. After every 7 days in various salt concentrations (0-150 mM), transgenic plants exhibited significant differences in leaf coloration compared to wild types. In particular, in 150 mM NaCl, leaves of the transgenic plant expressing the *codA* transgene remained green, whereas untransformed plants exhibited growth retardation at 100 mM NaCl. All of them appeared to grow better compared to control plants under the same salt stress, and leaves of *codA* transgenic lines are much larger than that of control plants under the same concentration of salt treatment. However, different lines showed some difference in salt tolerance, which could be due to different protein expression level of *CodA*.

SOD activity, malondialdehyde content and total chlorophylls in leaves were measured in transgenic plants during long-term salt exposure (150 mM NaCl) (Figs. 7A-C). SOD activity significantly increased after Salt stress 2 weeks, and SOD remained stably elevated over the measurement period (4 weeks) among the four transgenic plants lines. Among the lines, the SOD activity increased by 92.3% in the T1 transgenic plant leaves, which were exposure for 2 weeks, compared to leaves from untreated transgenic plants. The SOD activity began to decline during treatment with NaCl

after 2 weeks especially in the T1 transgenic plant. There was significant MDA accumulation after 7 days NaCl stress in all transgenic plants tested, reaching a peak of 23.23U.mg^{-1} protein after two weeks (64.1% higher than untreated transgenic plants), and then MDA content decreased rapidly especially in T1 transgenic plant, MDA remained stably elevated over the 4 weeks stress. Fig. 7C indicates that the contents of total chlorophylls in leaf discs were significantly decreased after salt stress (150mM NaCl). However, the different transgenic plants showed some difference under the same salt concentration. Over the course of 4 weeks salt stress, chlorophyll content gradually decreased, however, the T1 transgenic plant first decreased and then increased. Transgenic line T1, in particular, showed a large, 92.3% increase in the SOD content, while the MDA content and total chlorophyll content have no-significant changes after four weeks salt stress. These results suggest that increased accumulation of *CodA* can improve the salt-tolerance of transgenic plants, especially in the T1 transgenic plant line, and the difference of salt tolerance among transgenic lines was very significant, indicating the different insertion affecting the expression of genes. There also existed a difference among different stress duration of the same line.

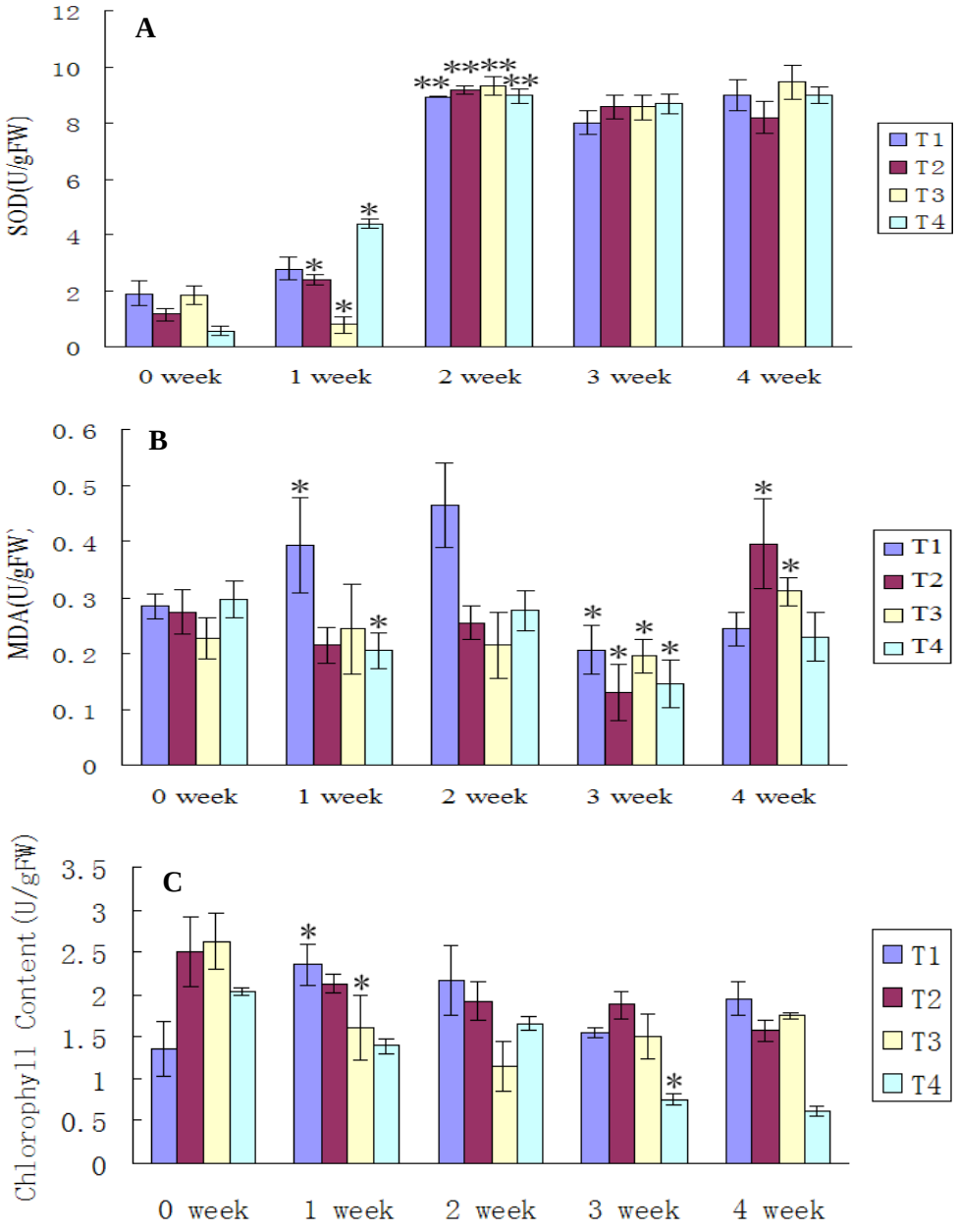

Fig. 7. Comparison of SOD activity, MDA content and chlorophyll content among *codA*-transformed tobacco plants. A: SOD activity comparison: B MDA content comparison; C: Chlorophyll content comparison; 0 week: before stress; 1, 2, 3, and 4 weeks: 150 mM NaCl treatment for 1, 2, 3, and 4 weeks.

Discussion

Many genes have been demonstrated to respond to drought, high salt levels cold stress, and the proteins encoded by these genes are thought to protect plant cells from these stressors (Xiong & Yang, 2003). In the present study, we were able to produce transgenic plants that were salt-tolerant by over-expressing a single gene, *codA*.

Transgenic plants are usually created by inserting a functional gene under the control of the 35S promoter from CaMV. The CaMV 35S promoter drives constitutive expression during all growth phase, however. The present work was aimed at targeted delivery of genes that enhance tolerance to salt stress, so we linked the gene to a promoter that enhances gene expression under conditions of (abiotic) osmotic stress, *Rd29A*, This should minimize unnecessary exposure of non-target plant tissues and plant consumers to the transgene. Indeed, the present study demonstrated that the *Rd29A* promoter was able to direct osmoprotectant gene expression when plants were exposed to salt stress, with the advantage that expression was absent or undetectable under natural growth conditions (Shinozaki & Shinozaki, 1993; Kasuga *et al*., 1999). The successful targeted expression of *codA* gene in *Nicotiana tabacum* could encourage further work aimed at engineering salt-tolerant transgenic plants. In experiments using the *Rd29A* promoter for direct foreign gene expression in tabacum, we are currently analyzing the expression profiles of an osmotant gene.

The stress-inducible *Rd29A* promoter is derived from *A. thaliana*. DRE (dehydration-responsive element) - and ABA- dependent binding sequences are found within the promoter region, and are shown to be independent of each other. The DRE/CRT (C-repeat) binding sequences are the target of the binding protein DREB1C. Rd29A activation by DREB1C is regulated by cold stress responses, yet salinity and drought stresses also activate Rd29A using alternative ABA-independent and -dependent transcription factors (Cong *et al.*, 2008).

In the present study, the recombinant vector pBS1305*Rd29codA* containing the *codA* gene was constructed under the control of the *Rd29A* promoter. The vector was then introduced into tobacco to obtain transgenic plants. To investigate the salinity tolerance of the *codA*-transgenic potato plants, two independent transgenic tobacco lines were selected via molecular identification. Both RT-PCR and physiological assays demonstrated that *codA* gene expression was induced by NaCl stress, given that the *codA* gene was controlled by the stress-induced promoter *Rd29A*. When the plants were exposed to NaCl stress, all transgenic plants showed a higher salinity tolerance than the control plants because of the *codA* gene expression. Transgenic plants exhibited significant differences in leaf coloration compared to wild types, SOD activity significantly increased after Salt stress two weeks, and SOD remained stably elevated among the four transgenic plants lines. However, the chlorophyll content of T1 transgenic plant first decreased and then increased when other plants gradually decreased. This result is similar to the findings obtained by Prasad, *et al*., (1995). These results demonstrate that engineering CK synthesis, especially through the use of a stress-inducible promoter such as *Rd29A*, is effective for imparting stress tolerance to the tobacco plant.

Soil salinity is a major factor in reducing plant growth and productivity. One means for enhancing salt tolerance is to improve the production of small osmolytes or stress proteins that protect against or reduce stress-induced damage (Zhu, 2001). Over-expression of the *codA* gene product also enhanced expression of the anti-oxidant enzyme SOD. These transgenic lines accumulated less MDA (Fig. 7B), possibly because the enhanced SOD activity protected membranes from peroxidation.

Acknowledgement

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References

Ahmad, K., M. Saqib, J. Akhtar and R. Ahmad. 2012. Evaluation and characterization of genetic variation in maize (*Zea mays* L.) for salinity tolerance. *Pak. J. Agri. Sci*., 49: 521-526.

Arnon, D.I. 1949. Copper enzymes in isolated chloroplasts polyphenoloxidase in *Beta vulgaris*. *Plant Physiology*, 24: 1-15.

Aubourg, S. and P. Rouze. 2001. Genome annotation. *Plant Physiol. & Biochem.*, 39: 181-193.

Bernard, T., M. Ayache and D.L. Rudulier. 1988. Restoration of growth and enzymatic activities of *Escherichia coil* Lac-mutants by glycine betaine. C.R.Acad.Sci. III. 307: 99-104.

Bohnert, H.J., P. Ayoubi, C. Borchert, R.A. Bressan, R.L. Burnap, J.C. Cushman, M.A. Cushman, M. Deyholos, R. Fischer, D.W. Galbraith, P.M. Hasegawa, M. Jenks, S. Kawasaki, H. Koiwa, S.K. Eda, L.B. Ha, C.B. Michalowski, E. Misawa, M. Nomura, N. Ozturk, B. Postier, R. Prade, S.H. Peng , Y. Tanaka, W. Hong and J.K. Zhu. 2001. A genomics approach towards salt stress tolerance. *Plant Physiol. & Biochem.*, 39: 295-311.

Bray, E.A., J.B. Serres and E. Weretilnyk. 2000. Responses to abiotic stresses. In: *Biochemistry and molecular biology of plants*. (Eds.): W. Gruissem, B. Buchannan and R. Jones. American Society of Plant Physiologists, Rockville, MD, pp. 1158-1249.

Cong, L., H. Zheng, Y. Zhang and T. Chai. 2008. Arabidopsis DREB1A confers high salinity tolerance and regulates the expression of GA dioxygenases in Tobacco. *Plant Sci.,* 174(2): 156-164.

Doyle, J.J. and E.E. Dickson. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem.* Bull., 19: 11-15.

Elizabeth E. Hood, Stanton B. Gelvin, Leo S. Melchers and Andre Hoekema. 1993. New Agrobacterium helper plasmids for gene transfer to plants. *Transgenic Research*, 2(4): 208-218.

Gan, S. and R.M. Amasino. 1997. Making sense of senescence: Molecular genetic regulation and manipulation of leaf senescence. *Plant Physiol*., 113: 313-319.

Giannopolitis, C.N. and S.K. Ries. 1977. Superoxide dismutase I, occurrence in higher plants. *Plant Physiol*., 59: 309-314.

Hasegawa, P.M., R.A. Bressan, J.K. Zhu and H.J. Bonhert. 2000. Plant cellular and molecular responses to high salinity. *Annual Reviews in Plant Physiology and Plant Molecular Biology*, 51: 463-499.

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

He, P.M., D.B. Zhang, W.Q. Liang, Q.H. Yao and R.X. Zhang. 2001. Expression of choline oxidase gene (*codA*) enhances salt tolerance of the tobacco. *Acta Biochimica et Biophysica. Sinica*, 33: 519-524.

Hitesh, K., G. Jitender, N. Karaba, N.M. Nataraja, U. Makarla and K.T. Akhilesh, 2009. Glycinebetaine-induced water-stress tolerance in *codA*-expressing transgenic indica rice is associated with up-regulation of several stress responsive genes. *Plant Biotech. J.*, 7: 512-526.

Holmberg, N. and L. Bülow. 1998. Improving stress tolerance in plants by gene transfer. 3(2): 61-66.

Holsters, M., D. de Waele, A. Depicker, E. Messens, M. Van Montagu and J. Schell. 1978. Transfection and transformation of Agrobacterium tumefaciens. *Mol. Gen. Genet*., 163: 181-187.

Ikuta, S., S. Imamura, H. Misaki and Y. Horiuchi. 1977. Purification and characterization of choline oxidase from *Arthrobacter globiformis*. *J. Biochem.*, 82: 1741-1749.

Incharoenskdi, A., T. Takabe and T. Akazawa. 1986. Effect of betaine on enzyme activity and subunit interaction of ribulose-1,5-bisphosphate carboxylase/oxygenase from *Aphanothece halophytica*. *Plant Physiol.*, 81: 1044-1049.

Ingram, J. and D. Bartels. 1996. The molecular basis of dehydration tolerance in plants. *Annual Reviews in Plant Physiology and Plant Molecular Biology*, 47: 377-403.

Ishizaki-Nishizawa, O., T. Fujii, M. Azuma, K. Sekiguchi, N. Murata, T. Ohtani and T. Toguri. 1996. Low-temperature resistance of higher plants is significantly enhanced by a nonspecific cyanobacterial desaturase. *Nat. Biotech.*, 14: 1003-1006.

Kalifa, Y., E. Perlson, A. Gilad, Z. Konrad, P.A. Scolinik and D. Bar-ar-ZVI. 2004. Overexpression of the water and salt stress-regulated Asr1 gene confers an increased salt tolerance. *Plant Cell Environ.*, 27: 1459-1468.

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

Kavi, K.P.B., Z. Hong, G.U. Miao, C.A.H. Hu and D.P.S. Verma. 1995. Overexpression of Δ´-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiol.*, 108: 1387-1394.

Kodama, H., T. Hamada, G. Horiguchi, M. Nishimura and K. Iba. 1994. Genetic enhancement of cold tolerance by expression of a gene for chloroplast ω-3 fatty acid desaturase in transgenic tobacco. *Plant Physiol.*, 105: 601-605.

McKersie, B.D., S.R. Bowley, E. Harjanto and O. Leprince. 1996. Water-deficit tolerance and field performance of transgenic alfalfa overexpressing superoxide dismutase. *Plant Physiol.*, 111: 1177-1181.

Mukhopadhyay, A., S. Vij and A.K. Tyagi. 2004. Overexpression of a zinc-finger protein gene from rice confers tolerance to cold, dehydration, and salt stress in transgenic tobacco. *Proc Natl. Acad. Sci., USA*, 101: 6309-6314.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant*, 15: 473-497.

Murata, N., R.S. Mohanty, H. Hayashi and G.C. Papageorgiou. 1992. Glycinebetaine stabilizes the association of extrinsic proteins with the photosynthetic oxygen-evolving complex. *Febs Letters*, 296: 187-189.

Papageorgiou, G.C. and N. Murata. 1995. The unusually strong stabilizing effects of glycinebetaine on the structure and function in the oxygen-evolving photosystem II complex. *Photosynthesis Res.*, 44: 243-252.

Papageorgiou, G.C., Y. Fujimura and N. Murata. 1991. Protection of the oxygen-evolving photosystem II complex by glycinebetaine. *Biochimica et Biophysica. Acta (BBA) - Bioenergetics*, 1057: 361-366.

Peever, T.L. and V.J. Higgins. 1989. Electrolyte leakage lipoxygenase, and lipid peroxidation induced in tomato leaf tissue by specific and nonspecific elicitors from *Cladosporium fulvum*. *Plant Physiol.*, 90: 867-875.

Prasad, K.V.S.K., P. Sharmila, P.A. Kumar and P.P. Saradhi. 2000. Transformation of *Brassica juncea* (L.) Czern with bacterial *codA* gene enhances its tolerance to salt stress. *Molecular Breeding*, 6: 489-499.

Robinson, S.P. and G.P. Jones. 1986. Accumulation of glycinebetaine in chloroplasts provides osmotic adjustment during salt stress. *Aust. J. Plant Physiol.*, 13: 659-668.

Sambrook, J., E.F. Fritsch and T. Maniatis. 1989. Molecular cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Santoro, M.M., Y. Liu, S.M. Khan, L.X. Hou and D.W. Bolen. 1992. Increased thermal stability of proteins in the presence of naturally occurring osmolytes. *Biochemistry*, 31: 5278-5283.

Shinozaki, Y.K. and K. Shinozaki. 1993. Arabidopsis DNA encoding two desiccation responsive rd29 genes. *Plant Physiol .*, 101: 1119-1120.

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

Xiong, L. and Y. Yang. 2003. Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. *Plant Cell*, 15: 745-759.

Xu, D.P., X.L. Duan, B.Y. Wang, B.M. Hong, T.H. David and R. Wu. 1996. 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.

Zhu, J.K. 2001. Plant salt tolerance. *Trends Plant Science*, 6: 66-71.

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