PROGRESS IN BIOLOGICAL SCIENCES

Responses of Transgenic Tobacco (*Nicotiana plambaginifolia*) Over-Expressing *P5CS* Gene Under *in vitro* Salt Stress

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ABSTRACT

Salinity is a major limiting factor for plant growth and development. To evaluate the impact of *P5CS* gene expression under *in vitro* salt stress condition, transgenic tobacco (*Nicotiana plumbaginifolia*) carrying *P5CS* gene and non-transgenic plants were treated with 0, 100, 150, 200 or 250 mM NaCl for 28 days. Proline content, lipid peroxidation and the activity of some antioxidant enzymes after salt treatment were measured. At 150 mM NaCl or higher, plant's leaf and root showed an increase in proline content significantly. Malondialdehyde (MDA) level in non-transgenic plants was considerably higher than the transgenic tobacco plant at 100 mM NaCl and higher. Activities of ascorbate peroxidase (APX) and catalase (CAT) was increased in transgenic as well as non-transgenic plants by increasing salt concentrations. However, transgenic plants showed higher level of antioxidant enzymes activity than non transgenic plants. Salinity tolerance of *Nicotine plumbaginifolia* transgenic *P5CS* gene might be closely related to the accumulation of proline, CAT and APX activity and consequently suppressed level of lipid peroxidation.

Keywords: Salt Stress, P5CS, Lipid Peroxidation, *Nicotiana plumbaginifolia*, Malondialdehyde, Transgenic.

Introduction

Drought and salinity are two major osmotic stresses that limit plant growth and development (Boyer, 1982). Around the world, approximately 20% of irrigated agricultural land is seriously affected by salinity (Flowers and Yeo, 1995). Understanding of the cellular and molecular responses of plants exposed to various biotic and abiotic stresses are important as they give hope for genetically modifying plants to cope with these stresses. There are many cellular mechanisms by which organisms modify the effects of environmental stresses, for instance accumulation of compatible osmolytes such as proline and glycin betaine (Diaz et al., 2005).

Proline accumulation under stress results from a stimulation of proline biosynthesis, (Szabados and Savoure, 2010) and it is mainly synthesized from glutamate by the enzyme pyrroline-5-carboxylate synthetase (P5CS) (Szekely et al., 2008). The proline synthesis pathway is present in both cytosol and chloroplast (Szabados and Savoure, 2010). Alternatively, proline can be produced from ornithine by the action of ornithine-δ-aminotransferase (OAT), (Delauney et al., 1993). In some plants, the arginase/OAT route seems to have an important role in stress-induced proline production (Xue et al., 2009). It can be considered that proline accumulation is due to a balance between activity P5CS (Δ-pyrroline-5-carboxylate synthetase) and proline oxidase (also called proline dehydrogenase, PDH) (Szekely et al., 2008).

In the last decade, several attempts were made to increase salt and other biotic stresses tolerance with proline accumulations in plants by transferring the genes associated with the proline biosynthetic pathway. Plant growth against salt stress was observed in a variety of way that were

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engineered by over production of proline. (Szekely et al., 2008; Xue et al., 2009).

It has been well documeted that, in the response of salt stress, plant increase osmotic potential within their cells by synthesizing and accumulating compatible osmolytes such as proline (Hanson and Hitz, 1980; Verbruggen and Hermans, 2008). Proline is known to acts as a compatible solute, an osmoprotectant, and a protective agent against cytosolic enzyme and cellular organelles in plants subjected to stresses resulted from drought and salinity (Delauney and Verma, 1993; Ehsanpour and Fatahian, 2003). In addition to acting as an osmoprotectant, proline also serves as a sink for energy to regulate redox potentials, as a hydroxy radical scavenge, and as a solute that protects macromolecules against denaturation (cited by Ehsanpour and Fatahian, 2003). 1-pyrroline-5-carboxylate synthase) *P5CS* (is a key enzyme in proline Biosynthesis pathway (Lee and Hwang, 2003). Expression of *P5CS* gene is a key factor to determine the synthesis of proline in plant (Lee and Hwang, 2003). Strong evidences indicate a positive correlation between accumulation of proline and salt tolerance in plants (Hu et al., 1992; Sanchez et al., 2010). Our previous study has indicated that in *Nicotiana tabacum* cv. Wisconsin as a model plant, over-expression of *P5CS* gene resulted in increasing of salt tolerance compare with control plant (Razavizadeh and Ehsanpour, 2009). It has been well shown that salt stress increase activity of antioxidant systems including enzymatic as well as non-enzymatic antioxidants (Abbasi and Komatsu, 2004). Since over-expression of proline in plants exposed to salt stress can increase the scavenging of ROS we are interested in to understand the stability of transformation and the responses of transgenic plants over a long term period of time. Therefore, the aim of the current study is to test T1 plants versus non transgenic (F1) *Nicotiana plumbaginifolia* plants were tested to determine the consequence of over-expression of p5CS gene in proline accumulation, CAT and APX activity and lipid peroxidation.

Materials & Methods

Plants Samples and NaCl Treatment

In our previous study, a coding sequence of the *P5CS* gene from moth bean (*Vigna aconitifolia)*, GenBank Accession number M92276, (Hu et al. 1992) was digested with *Mul* I and *EcoR* I, and ligated between the CaMV 35S promoter and Nos terminator in the pGA748 vector. The constructed pGAP5CS vector was introduced into *Agrobacterium tumefaciens* (LBA4404). This construct was kindly provided by Professor Cheol Ho Hwang, School of Bioresource Science, Dankook University, Cheonan, South Korea. Tobacco leaf segments were used for transformation. Transgenic plants were regenerated and seeds of transgenic tobacco (*Nicotiana plumbaginifolia*) from T1 line and non-transgenic (Fig. 1) were sterilized using 10% (v/v) sodium hypochloride and washed with sterile distilled water thoroughly and then placed on MS solid medium (Murashige and Skoog, 1962). All cultures were subsequently grown in a growth chamber under white fluorescent light (600 $\mu molM^{-2}S$; 16 h light ; 8 h dark) at 25°Cand 90% humidity. Two months after germination, 18 individual plants in three replications from transgenic and non-transgenic (wild type) lines were sub cultured and then 25-28 days old rooted plants were transferred to liquid MS medium containing 0, 100, 150.200 and 250 mM NaCl.

Determination of Proline Content

The concentration of proline was measured according to the method of Bates et al. (1973). 500 mg of frozen plant material (leaf and root) l was homogenized with 10 ml of 3% (W/V) sulphosalicylic acid and passed through whatman filter paper No. 1. In that case, 1 ml of the extract was reacted with 1 ml glacial acetic acid and 1 ml ninhydrin 1.25 g ninhydrin warmed in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid until dissolved) in 100°C water bath. The reaction mixture was extracted with 4 ml toluene. The Chromophore-containing toluene was measured at 520 nm against toluene as blank using spectrophotometer (Beckman, fullerton, CA, USA). Proline concentration was determined using calibration curve as μmol proline g^{-1} FW.

Lipid Peroxidation

The level of lipid peroxidation in leaf samples was determined based on malondialdehyde

(MDA) content according to the method of (Moore and Roberts, 1998). Content of MDA is an end product of lipid peroxidation. A leaf segment (200 mg) was separately homogenized with 5 mL trichloroacetic acid (TCA). The homogenate was centrifuged for 5 min at 10000rpm then the supernatant was mixed with thiobarbituric acid (TBA) (0.5% in 20% (w/v) TCA. The reaction mixture was incubated at 95°C in the water bath for 30 min and the reaction was finished in an ice bath. MDA concentration was calculated from the absorbance at 532 nm and measurements were corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The concentration of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Enzyme Extractions and Assays

100 mg of leaves samples were homogenized with 1 ml ice cold phosphate buffer saline (pH:7.8) containing 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, 2.7 mM KCl, 140 mM NaCl and 1% (w/v) polyvinylpoly-pyrolidone (PVPP). Homogenates were centrifuged at 4°C for 20 min at 13000 rpm, and supernatants were used for determination of protein content and enzyme activity.

Catalase (CAT; EC 1.11.1.6)

Catalase activity was estimated according to Aebi, (1984); in which the initial rate of disappearance of H_2O_2 at 240 nm/100 µL enzyme extract was reacted with 900 µl phosphate-buffer saline (pH: 7) containing 10 mM H_2O_2. The concentration of oxidized catalyse was calculated using extinction coefficient (0.0394 $mM^{-1}cm^{-1}$). One unit of catalase was defined by µmol H_2O_2 destruction per minute.

Ascorbate Peroxidase (APX; EC 1.11.1.11)

Ascorbate peroxidase activity was measured according to Nakano and Asada. (1981). The assay depends on the decrease in absorbance at 290 nm as ascorbate was oxidized the reaction mixture contained 50 mM phosphate buffer saline (pH: 7), 0.5 mM ascorbate, 0.1 mM EDTA Na_2, 1.2 mM H_2O_2 and 0.1 mm of enzyme extract in a final assay volume of 1 ml. The concentration of oxidized ascorbate was calculated using extinction coefficient of 2.8 $mM^{-1}cm^{-1}$. One unit of APX was defined as 1 $\mu molmL^{-1}$ ascorbate oxidized per minute.

Statistical analysis

All experiments were carried out with at least three replications (three plant samples) from tissue culture containers of transgenic as well as non-transgenic (wild type) plants. The physiological and biochemical parameters were statistically analyzed using ANOVA andmean data were compared using Tukey test.

Results

Proline Content

The proline content increased in leaf and root of transgenic and non-transgenic plants after salt treatment. Free proline content in leaves of both tobacco plants exposed to 200 mM NaCl showed the highest level compare to untreated plants. Transgenic plants showed significantly higher proline than non-transgenic plants (Fig. 1). Our result showed that at 150, 200, 250 mM NaCl, similar to leaf, accumulation of proline in roots of transgenic plants was much higher than non-transgenic plants (Fig. 2)

Lipid peroxidation

The content of MDA in leaves was increased by rising of NaCl concentration. The highest amount of MDA was observed at 200 and 250 mM NaCl. The MDA content in non-transgenic plants was significantly higher than transgenic plants in all salt treatments (Fig. 3).

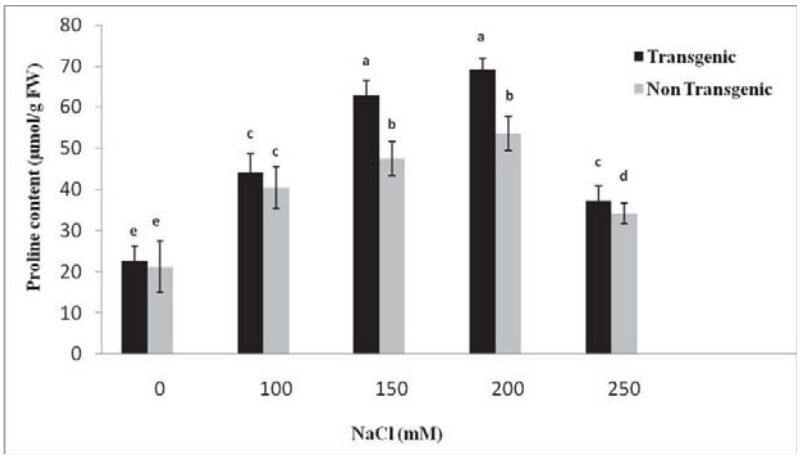

Figure 1. Effect of NaCl on the proline contents in leaves of *Nicotiana plumbaginifolia* plants. Similar letters are not significant (P<0.05) base on Tukey test.

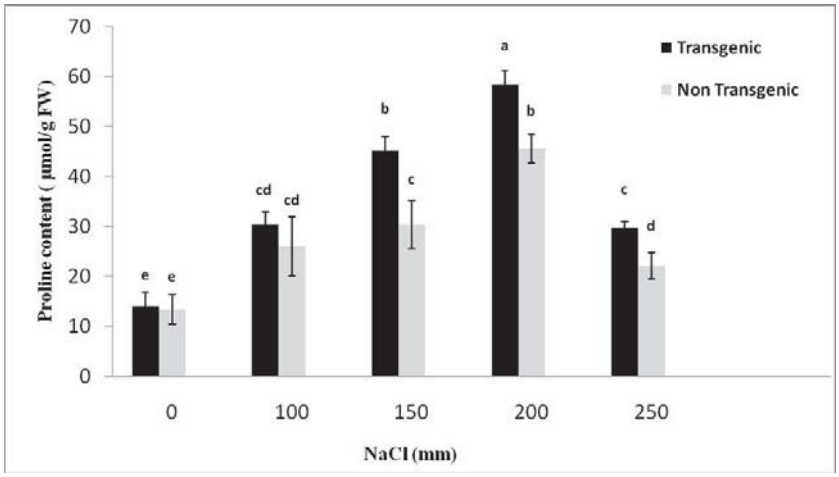

Figure 2. Effect of NaCl on the proline contents in roots of *Nicotiana plumbaginifolia*plants. Similar letters are not significant (P<0.05) base on Tukey test.

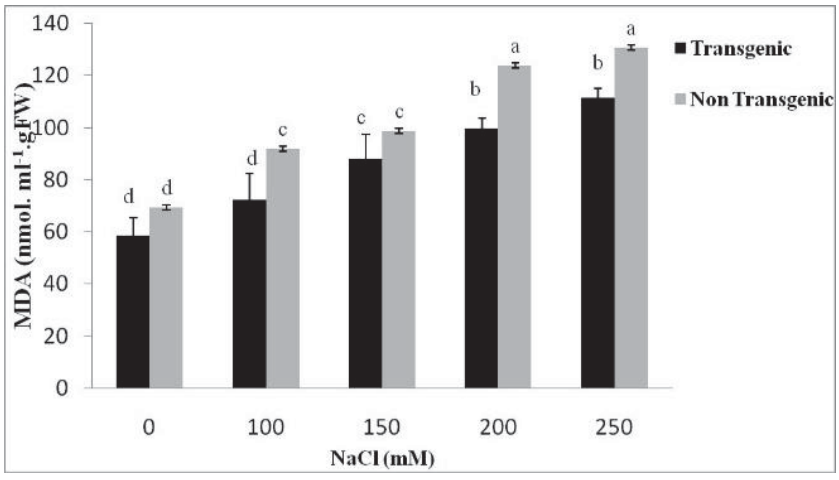

Figure 3. Effect of NaCl concentration on the malondialdhyde (MDA) contents in leaves of *Nicotiana plumbaginifolia* plants. Similar letters are not significant (P<0.05) base on Tukey test.

Activity of CAT

General pattern of CAT activity showed that by increasing salt concentration in the medium the CAT activity was increased except at 250 mM. Maximum activity of CAT in transgenic plants was observed in 200 mM NaCl. However, in non-transgenic plants 150 mM NaCl illustrated the highest activity of CAT enzyme (Fig. 4).

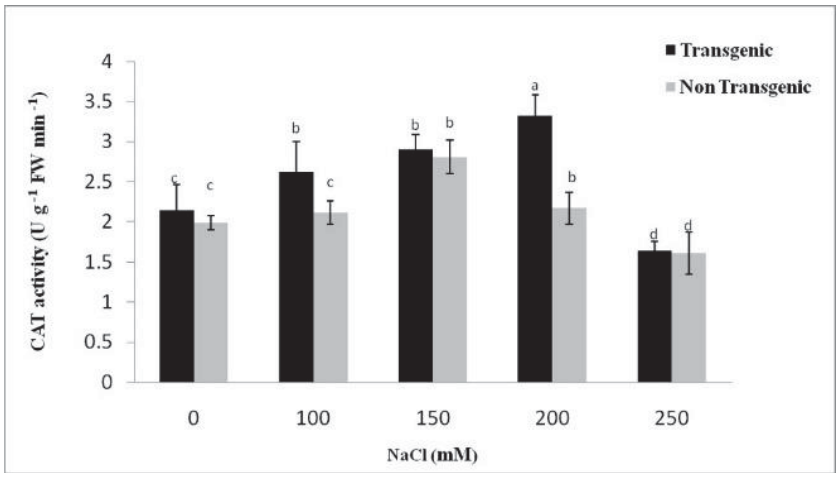

Figure 4. Effect of NaCl concentration on CAT activity in leaves of *Nicotiana plumbaginifolia* plants. Similar letters are not significant (P<0.05) base on Tukey test.

Activity of APX

APX activity in salt treated plants leaves was similar to CAT. Both transgenic and non-transgenic plants showed increasing level of APX when salt concentration was increased in the medium. Similar to transgenic plant had the highest APX activity at 200 mM while non-transgenic plant demonstrated the high level of APX activity at 100 and 150 mM NaCl without any significant difference (Fig. 5).

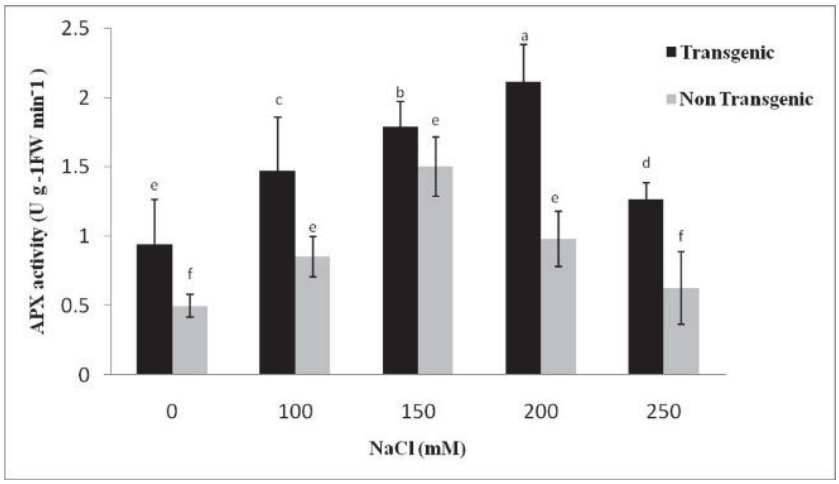

Figure 5. Effect of NaCl concentration on APX activity in leaves of *Nicotiana plumbaginifolia* plants. Similar letters are not significant (P<0.05) base on Tukey test.

Discussion

Salinity is a major factor in reducing the growth and productivity of plants and involves three different responses: 1) dehydration of the cells as a result of low water potential, 2) nutritional imbalance caused by interference by ions in the uptake and translocation of essential nutrients, and 3) toxicity as a result of accumulation of large amounts of Na^+ and Cl^- ions in the cytoplasm (Ehsanpour and Fatahian, 2003). To determine the effect of proline accumulation on plant growth and development, we measured proline content in both transgenic and non-transgenic plants treated with NaCl in the medium. Significant difference between non transgenic and transgenic plants over-expressing *P5CS* gene was observed. Over-expression of P5CS gene resulted in accumulation of much more proline compare to non-transgenic plants. Proline accumulation increased salt tolerance in tobacco plants (Razavizadeh, Ehsanpour, 2009). Proline as an osmoprotectant has different functions such as protecting enzyme and proteins, it also has a role as antioxidant. Similar function of proline has also been previously reported. Proline contribute to osmotic adjustment and protection of macromolecules during dehydration and as a hydroxyl radical scavenger and reducing toxic ion uptake. Evidence supporting the role of proline during salt stress was obtained based on salt tolerance in transgenic tobacco plants with enhance levels of proline biosynthesis and salt tolerance of *Arabidopsis* with suppressed level of proline degradation (Hare et al., 1998; Zhu, 2000). The increase of proline content decreases the harsh effect of salt stress on plant functions (Delauncy and Verma, 1993). Recent reports indicate that the accumulation of other osmoprotectants, such as mannitol, fructan or terhalose may increase tolerance to water deficit (kishor et al., 1995).

During salt stress Δ-pyrroline-5-carboxylate dehydrogenase (P5CDH) is stimulated by salt. It indicates that PDH activity could play a role in the inhibition of proline during salt stress (Hellmann et al., 2000). Decreasing of proline level may occur by feedback inhibition mechanism too (Deuschle et al., 2001). The production of proline seems to improve the salinity tolerance of transgenic tobacco *Nicotiana plumbaginifolia* plants to a certain level of 0, 100, 150, 200, 250 mM NaCl treatment and the potential use of these transgenic plants. Our result indicated that over-expression of *P5CS* gene improved the salinity tolerance by different strategies such as, high proline level, antioxidant enzyme activity and ROS scavenging process. It has been shown that Reactive Oxygen Species (ROS) are formed during salt and osmotic stress. One of the consequences of ROS production is lipid peroxidation of the membrane (Davis, 1995). Increasing of MDA concentration as a criteria for membrane damage was reported under salt and drought stress (Yan, 2009). MDA, as a lipid peroxidation product, has been used widely to assess the level of free radicals in living cells (Kunert and Ederer, 1985; Guether et al., 2009). It is concluded that MDA levels increased significantly with NaCl concentration in both transgenic and non-transgenic tobacco plants, but MDA content in non-transgenic were higher than of transgenic. It can be speculating that, transgenic plant produced more proline and accumulated less MDA than non-transgenic plants. These results clearly showed a role of proline in scavenging of free radicals in tobacco plants exposed to salinity. On the other hand, increase in MDA level under of salt treatment might also be correlated with inadequate activity of CAT to scavenge ROS in tobacco leaves. Some studies have shown a positive relation between low peroxidation damage and a more efficient activity of antioxidative system in cotton and tomato (Shalata and Tal, 1998; Gossett et al., 1994). Similar pattern might be considered in our plants. ROS are produced in vital processes such as photorespiration, photosynthesis and respiration. For protection against ROS, plants contain antioxidant enzymes as well as a wide a range of non-enzymatic antioxidants (Saibo et al., 2009). Reduction of oxidative damage could provide enhanced plant resistance to salt stress (. Sanchez et al., 2008). In plant cells salt stress induces generation of reactive oxygen species (ROS) such as single oxygen ($^1O_2$), Superoxide anion (O_2°), Hydrogen peroxide (H_2O_2) and Hydroxyl radical (OH°) that alter the lipids, proteins, enzymes, DNA and membrane structure and function (Hernandez et al., 2000). Antioxidant enzymes are involved in the detoxification of ROS. For example, superoxide

dismutases (SOD) convert superoxid to H_2O_2 in different sub cellular compartments; H_2O_2 is then scavenged by Catalase (CAT), Ascorbate peroxidase (APXs) (Bowler, 1992). Plants use antioxidants such reduced glutathione (GSH) and different enzymes such as superoxide dismutases (SOD), CAT, APX glutathione-s-transferases (GST) and glutathione peroxidas (GPX) to scavenge ROS. APX and CAT activity was studied in both tobacco plants transgenic and non-transgenic. The down regulation of CAT and APX by salt stress (250 mM NaCl) may indicate that the plant is not able to maintain protection against oxtidative oxygen under salt stress particularly at high salt concentration. Such a decrease may be due to some stress induced damages to the enzyme. From the results, it is concluded that increase in activity of CAT and APX at 0, 100, 150, 200, 250 mM NaCl in transgenic plant may be supported by the ability of proline to maintain higher catalase and APX activity under stress. Based on the present data we can speculate that P5CS gene in F1 tobacco plants are stable and transformed plants can be used for further than one generation.

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References

1. Abbasi FM, Komatsu SA (2004) Proteomic approach to analyze salt responsive proteins in rice leaf sheath. Proteomics 4, 2072-2081
2. Aebi H (1984) Catalase *in vitro* Methods in Enzymology 105, 121-126
3. Bates L, Waldren RP, tear IP (1973) Rapid determination of free proline for water stress studies. Plant *Soil* 39, 205-207
4. Bowler C Montagu M, Inze D (1992) Superoxide dismutase and stress tolerance. Plant of Molecolar Biology 43, 83-116
5. Boyer JS (1982) Plant productivity and environment. Science 218, 443-448
6. Davies KJ (1995) Oxydative stress: the paradox of aerobic life. Biochem Soc Symp 61, 1-31
7. Delauney AJ , Hu CA, Kishor PB, Verma DP (1993). Cloning of ornithine delta-aminotransferase cDNA from *Vigna aconitifolia* by trans-complementation in *Escherichia coli* and regulation of proline biosynthesis. Journal of Biological Chemistry 268, 18673–18678
8. Delauney AJ, Verma DPC (1993) Proline biosynthesis and osmoregulation in plants. Plant Journal 4, 215-223
9. Deuschle K, Funck D, Hellmann H, Däschner K, Binder S, Frommer WN (2001). A nuclear gene encoding mitochondrial Δ1-pyrroline-5-carboxylate dehydrogenase and its potential role in protection from proline toxicity. Plant Journal 27, 345–355
10. Díaz P, Borsani O, Márquez A, Monza J (2005) Osmotically induced proline accumulation in *Lotus corniculatus* leaves is affected by light and nitrogen source. Plant Growth Regulation 46, 223–232
11. Ehsapour AA, Fatahian N (2003) Effect of salt and proline on *Medicago sativa* callus. Plant Cell tissue and organ culture 73, 53-56
12. Flowers TJ, Yeo AR (1995) Breeding for salinity resistance in crop plants . Journal of Plant Physiology 22, 875-884
13. Gosset DR, Millholon EP, Lucas MC (1994) Changes in antioxydant levels in response to NaCl treatment salt tolerant and sensitive cultivars of cotton (*Gossypium hirsutum* L.) Crop Science Direct 34, 706-714
14. Guether M, Balestrini R, Hannah M, He J, Udvardi MK, Bonfante P (2009) Genome-wide reprogramming of regulatory networks, transport, cell wall and membrane biogenesis during arbuscular mycorrhizal symbiosis in *Lotus japonicus*. New Phytologist 182, 200–212
15. Hanson AD, Hitz WD (1980) Metabolism response of mesophytic plants to water deficits. Plant Physiology 33, 163-203
16. Hare Pm, Cress WA, Van Staden J (1998) Dissecting the roles of osmolyte accumulation during stress. Plant Cell Environment 21, 535-553

17. Hellmann H, Funck D, Rentsch D, Frommer WB (2000) Hypersensitivity of an *Arabidopsis* suger signaling mutant toward exogenous proline application. Plant Physiology 122, 357-367

18. Hernandez JA, Jimenez A, Mullineaux P, Sevilla F (2000) Tolerance of pea (*Pisum sativum* L.) to long term salt stress in associated with induction of antioxidant defenses. Plant Cell Environment 23, 853-862

19. Hu cha, Delauney aj, Verma D (1992) A bifunctional enzyme (Δ1 pyrroline-5-carboxylate synthetase) catalyzes the first two steps in proline biosynthesis in plants. Plant Biology 89, 9354–9358

20. Kishor B, Hong Z, Miao G, Hu C, Verma D (1995) Overexpression ofΔ- Pyrroline-5-carboxylate synthetase increase proline productionand confers osmotolerance in transgenic plants . Plant Physiology 108, 1387-1394

21. Kunert KJ, Ederer M (1985) Formation of reactive oxygen species and changes in antioxydative systems leaf aging and lipid peroxidation: The role of the antioxidants vitamin C and E. Physiologia Plantarum 65, 85-88

22. Lee DJ, Hwang CH (2003) Proline accumulation and P5CS (Δ- Pyrroline-5-carboxylate synthetase) gene expression in response to salt stress in Zoysiagrasses. Journal Crop Science 48, 20-24

23. Moore K, Roberts LJ (1998) "Measurement of lipid peroxidation". Free Radical Research 28, 659–71

24. Murashige T, Skoog FA (1962) Revised medium for rapid growth and bioassays with tobacco cultures. Plant Physiology 159, 473-479

25. Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiology 22, 867–880

26. Razavizadeh R, Ehsanpour AA (2009) Effects of salt stress on proline content, expression of delta-1-pyrroline-5-carboxylate synthetase, and activities of catalase and ascorbate peroxidase in transgenic tobacco plants. Biological Letters 46, 9–21

27. Saibo NJM, Lourenço T, Oliveira MM (2009) Transcription factors and regulation of photosynthetic and related metabolism under environmental stresses. Annals of Botany 103, 609–623

28. Sánchez DH, Lippold F, Redestig H, Hannah MA, Erban A, Krämer U, Kopka J, Udvardi MK (2008) Integrative functional genomics of salt acclimatization in the model legume *Lotus japonicus*. Plant Journal 53, 973–987

29. Sanchez DH, Szymanski J, Erban A, Udvardi MK, Kopka J (2010). Mining for robust transcriptional and metabolic responses to long-term salt stress: a case study on the model legume *Lotus japonicus*. Plant, Cell & Environment 33, 468–480

30. Sawahel WA, Hassan AH (2002) Generation of transgenic wheat plants producting high levels of the osmoprotectant proline. Biotechnology Letters 24, 721-725

31. Shalata A, Mittova V, Volokita M, Guy M, Tal M (2001) Response of cultivated tomato and its wild salt tolerant relative *Lycopersicom pennelli* to salt dependent oxidative stress: the root antioxidative system. Physiologia Plantarum 122, 487-494

32. Szabados L, Savouré A (2010) Proline: a multifunctional amino acid. Trends in Plant Science 15, 89–97

33. Székely G, Ábraham E, Cséplő A, Rigó G, Zsigmond L, Csiszár J, Ayaydin F, Strizhov N, Jásik J, Schmelzer E et al. (2008) Duplicated P5CS genes of *Arabidopsis* play distinct roles in stress regulation and developmental control of proline biosynthesis. Plant Journal 53, 11–28

34. Verbruggen N, Hermans C. (2008) Proline accumulation in plants: a review. Amino Acids 35, 753–759

35. Wang Z-Q, Yuan Y-Z, Qu J-Q, Lin Q-H, Zhang C-F. (2007) Glutamine synthetase and glutamate dehydrogenase contribute differentially to proline accumulation in leaves of wheat (*Triticum aestivum*) seedlings exposed to different salinity. Journal of Plant Physiology 164, 695–701

36. Xue X, Liu A, Hua X (2009) Proline accumulation and transcriptional regulation of proline biosynthesis and degradation in *Brassica*

napus. BMB Reports 42, 28–34

37. Yan L (2009). Physiological responses of tomato seedlings (*Lycopersicon esculentum*) to salt stress. Modern Applied Science 3, 171-176

38. Zhu B, Su J, Chang M, Verma DPS, Fan YL, Wu R (1988) Over-expression of a pyroline -5-carboxylate synthase gene and analysis of tolerance to water and salt stress in transgeneic rice. Plant Science 139, 41-48

39. Zhu J (2000). Genetic analysis of plant salt tolerance using *Arabidopsis*. Plant Physiology 124, 941-948