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The effect of water deficit and excess copper on proline metabolism in *Nicotiana benthamiana*

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Abstract

Fluctuation in proline content is a widespread phenomenon among plants in response to heavy metal stress. To distinguish between the participation of water deficit and copper on changes in proline metabolism, potted plants and floating leaf discs of tobacco were subjected to $CuSO_4$ treatments. The application of copper increased the proline content in the leaves concomitantly with decreased leaf relative water content and increased abscisic acid (ABA) content in the potted plant. Excess copper increased the expression of two proline synthesis genes, pyrroline-5-carboxylate synthetase (P5CS) and ornithine aminotransferase (OAT) and suppressed proline catabolism gene, proline dehydrogenase (PDH). However, in the experiment with tobacco leaf discs floating on $CuSO_4$ solutions, the excess copper decreased proline content and suppressed the expression of the P5CS, OAT and PDH genes. Therefore, proline accumulation in the potted tobacco plants treated with excess Cu treatment might not be the consequence of the increased copper content in tobacco leaves but rather by the accompanied decrease in water content and/or increased ABA content.

Additional key words: abscisic acid, gene expression, tobacco.

Introduction

Numerous reports have shown that expose to excess heavy metals, such as Cu, Zn, Co, Cd, Pb, Cr, Hg and Ni, can induce proline accumulation in plants (Saradhi and Saradhi 1991, Schat *et al.* 1997, Mehta and Gaur 1999, Nagoor and Vyas 1999, Tripathi and Tripathi 1999, Oncel *et al.* 2000, Talanova *et al.* 2000, Chen *et al.* 2001, Siripornadulsil *et al*. 2002, Wu *et al*. 2005). Proline accumulation is a well-known phenomenon among plants in response to water deficit and has often been associated with the osmoregulation (Chen and Kao 1993, Delauney and Verma 1993, Girousse *et al.* 1996, Armengaud *et al*. 2004, Walker *et al*. 2010). Numerous metal-tolerant plant species contained elevated contents of proline in different plant parts (for a review see Sharma and Dietz 2006). Many studies have shown that under heavy metal stress the water transport in plant decreased and resulted in water deficit in the shoots (Kastori *et al.* 1992, Haag-Kerwer *et al.* 1999, Chen *et al.* 2004). Thus, it is reasonable to assume that both heavy metal ions and water deficit participate on proline accumulation in plants. However, fluctuating pattern of proline accumulation under Zn and Cu treatments in wheat and the decrease of proline content under Cu treatments in *Mesembryanthemum crystallinum* were also reported (Bassi and Sharma 1993, Thomas *et al.* 1998).

The mechanism of proline accumulation could be due to increased proline synthesis, decreased proline catabolism or increased soluble protein degradation (Chen *et al*. 2001). There are two proline synthetic pathways in higher plants (Charest and Phan 1990). One is the conversion of glutamate *via* the action of pyrroline-5-carboxylate synthetase (P5CS) (Voetberg and Sharp

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Abbreviations: ABA - abscisic acid; OAT - ornithine aminotransferase; P5CS - pyrroline-5-carboxylate synthetase; PDH - proline dehydrogenase; RWC - relative water content.
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1991, Turchetto- Zolet *et al*. 2009, Thippeswamy *et al*. 2010), while the other is the conversion of ornithine (Canas *et al*. 2008, Funck *et al*. 2008) *via* ornithine aminotransferase (OAT). Conversely, proline can be catabolized to glutamate *via* the action of proline dehydrogenase (PDH; Kiyosue *et al.* 1996, Peng *et al.* 1996, Verbruggen *et al.* 1996). Studies on the pattern of gene expression have revealed proline accumulation in many stress conditions. For examples, treatments of salt and water stress stimulated the proline accumulation along with the increase of P5CS mRNA level (Hu *et al.* 1992, Savoure *et al.* 1995, Verbruggen *et al.* 1996, Yoshiba *et al.* 1997). During and after osmotic stress, the level of proline seems to be controlled *via* the reciprocal regulation of P5CS and PDH genes (Peng *et al.* 1996). Furthermore, there is evidence that salt stress also down-regulates PDH mRNA accumulation. (Kiyosue *et al.* 1996, Peng *et al.* 1996, Verbruggen *et al.* 1996). Though the accumulation of proline can be due to the increase of P5CS mRNA production (Hu *et al.* 1992, Delauney and Verma 1993, Fujita *et al.* 1998), it has also been proposed that the accumulation of proline could result from the release of the feedback inhibition of P5CS (Delauney and Verma 1993). To date, little attention has been paid to the ornithine pathway. Kandpal and Rao (1982) found that OAT activity in *Eleusine coracana* leaves increased under water stress. The ornithine pathway as well as the glutamate pathway contribute to proline biosynthesis under salt stress (Hervieu *et al.* 1995). In addition, Roosens *et al.* (1998) noted that salt treatment increased OAT mRNA level in *Arabidopsis*, but the rate of increase was slower than that of the increase of P5CS mRNA.

Although there are many studies on the pattern of stress-induced gene expression related to proline metabolism, most have focused on the whole plant proline content, which did not allow to distinguish the effect of water deficit from direct effect of NaCl or heavy metals treatment. Here we compare the results of potted plants and floating leaf discs to distinguish between the participation of water deficit and excess copper on proline metabolism. We also describe the expression of three proline metabolism related genes, P5CS, OAT and PDH, under Cu stress.

Materials and methods

Tobacco (*Nicotiana benthamiana* Domin) plants were grown in 0.3-dm^3 pots containing the mixture of *Vermiculite*, *Perlite* and organic matter (3:1:1) for 28 d in a greenhouse at controlled temperature of 28 °C and natural irradiance. Then 100 cm^3 of 0 to 80 mM $CuSO_4$ solutions were added into the pots. The added Cu ion was absorbed initially by the potting material, after which it was released gradually to create a dynamic available Cu for plant. Therefore, the Cu content in tobacco leaves was measured as the indicator of the degree of Cu stress (Fig. 1*A*). Water stress treatment just resulted from simply cessation of watering. For leaf disc experiments, a puncher with a diameter of 1.2 cm was used to cut the discs from mature leaves. Leaf discs were air dried in Petri dishes or transferred to Petri dishes containing the incubation medium supplemented with Cu (0 to 5 mM) for 24 h. Throughout the different treatments, tobacco leaves (0 to 6 d) as well as excised leaf discs (0 to 24 h) were collected, quickly frozen in liquid nitrogen and stored in a -80 ºC for further analysis.

Relative water content (RWC) was calculated as follows: RWC [%] = [(fresh mass - dry mass)/(water saturated mass - dry mass)] × 100, where water saturated mass was determined after soaking in distilled water for 8 h and dry mass after drying at 70 ºC for 72 h (Chen *et al.* 2004).

For the determination of Cu content, plant material was digested in a microwave oven with 10 cm^3 70 % HNO_3 solution, then analyzed by inductively coupled plasma-atomic emission spectrometry (*JY124*, *Jobin-Yvon*, Edison, NJ, USA) according to Chen *et al.* (2004). The content of proline was determined according to the method of Bates *et al.* (1973). Frozen tissues were homogenized using 3 % sulfosalicylic acid and boiled with acetic acid and ninhydrin for 1 h, and then the absorbance at 520 nm was determined. For protein determination, frozen tissues were homogenized in 50 mM sodium phosphate buffer (pH 6.8), then were centrifuged at 17 600 *g* for 20 min. Next, the supernatants were analyzed for protein content as described by Bradford (1976). According to the method reported by Chen *et al.* (2001), ABA was extracted from leaf samples, which were homogenized in an extraction solution (80 % methanol containing 2 % glacial acetic acid), passed through a polyvinylpyrrolidone column, and subsequently thorough C18 cartridges. The elutes were dried by vaccum-evaporation and dissolved in Tris-buffered saline. Enzyme-linked immunosorbent assay was conducted by immunoassay detection kit (*PGR-1*) from *Sigma* (St. Louis, MO, USA).

Aliquots containing 10 µg total RNA were separated by electrophoresis on a formaldehyde-containing agarose gel (Sambrook and Russell 2001) and subsequently blotted onto a nylon membrane (*Gene Screen Plus*, *NEN*, Boston, MA, USA). The probes for RNA blotting analysis were three tomato proline synthesis- and catabolism-related genes cloned in this study using RT-PCR and TOPO cloning of RNA from tomato leaves and the method was originally described by Napoli *et al.* (1990) with minor modification (Pang *et al*. 1997, Jan

et al. 1999, 2000). The degenerated primers for RT-PCR (Table 1) were designed from the nucleotide sequences of *P5CS*, *OAT* and *PDH* genes from GenBank using *BLAST* (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the *Clustal* alignment of *MegAlign* (*Dnastar*, Madison, USA). The accession numbers of cloned tomato *P5CS*, *OAT* and *PDH* partial gene fragments were AY897574, AY897573 and AY897575, respectively. These three cDNA clones were radio-labeled as probes by using α-^{32}P-dATP (0.37 GBq mmol^{-1}) using random hexamer priming (Feinberg and Vogelstein 1983). RNA hybridization was taking place at 60 ºC for 16 h. Washing was performed twice at 2× SSC, 1 % SDS at 60 ºC for 30 min, followed with a wash at 0.5 × SSC, 1 % SDS for 60 ºC for 30 min and an additional wash at 0.1× SSC at 60 ºC for 10 min.

At least three replicates were conducted in all the treatments. Every experiment was repeated at least three times, and one of them was chosen for the graphical representation. Duncan's multiple range test was performed to indicate the significant difference among treatments ($P < 0.05$)

Table 1. Oligo-primers with their respective sequences used in this study. TACGTA: restriction enzyme site of *Sna*BI, GCGGCCGC: restriction enzyme site of *Not*I.

Name		Sequence
FJJ2002-20	upstream primer of OAT	5' TATATAGCGGCCGCGTGC(C/T)CACAATTACCA
FJJ2002-21	downstream primer of OAT	5' CGACGATACGTATCATT(A/G)TCACA(A/G)CTCAT
FJJ2001-117	upstream primer of P5CS	5' ACACACTACGTACCATGGTTGG(A/G)AC(A/T)GC(A/T)GTTGT
FJJ2001-118	downstream primer of P5CS	5' TCAGACGCGGCCGCATCATT(C/T)TCATT(G/T)AA
FJJ2001-73	upstream primer of PDH	5' ACGTTACGTACCATGGTTGATGCGGAAGACACAA
FJJ2001-74	downstream primer of PDH	5' ATATAGCGGCCGCG(A/C)CATACCATATAGCTG

Results

To evaluate water deficiency and excess copper stress on proline metabolism, the RWC and Cu content were monitored under excess Cu and water deficient treatment. Leaf Cu content increased in accordance with the increase of Cu dosage applied to the roots (Fig. 1*A*). After cessation of watering, RWC in tobacco leaves decreased more in those treated with Cu, except 20 mM Cu (Fig. 1*B*). The decrease of RWC induced by 40 mM Cu treatment occurred at the third day of treatment, then remained constant afterward. The decrease of RWC also occurred under 80 mM Cu indicating severe wilting of the whole plant (Fig. 1*B*).

The excess of Cu increased proline content of tobacco leaves (Fig. 1*C*) and the proline accumulation reached a plateau at 80 mM Cu. As expected, water deficit increased proline content dramatically (Fig. 1*C*). With regard to the possibility that proline may have been released from proteins, the leaf protein contents were monitored. However, no difference was found in protein content among control, water deficit and 20 mM Cu (Fig. 1*D*). In contrast, 40 and 80 mM Cu decreased protein content from the 3rd d of treatment. It was noted that potted plants submitted to Cu treatment and/or water deficit indicated marked increase in ABA content (Fig. 1*E*).

As concern gene activities, low 20 and 40 mM Cu, as well as water deficit, increased the expression of *P5CS* and *OAT* genes in the potted plants (Fig. 2*A*). However, the increase in *P5CS* and *OAT* expression was lower in response to Cu treatments than to water deficit. Furthermore, the increasing expression of both genes declined at the day 6 of 80 mM Cu treatment. Concerning the proline catabolism gene, both water deficiency and Cu treatment decreased *PDH* expression in potted plants (Fig. 2*A*).

The Cu content in the leaf discs also increased in accordance with the Cu concentrations in the incubation medium (Fig. 3). The Cu contents in leaf discs treated with 0.01, 0.1 and 0.5 mM Cu (Fig. 3) were similar to those of potted plants treated with 20, 40 and 80 mM Cu, respectively (Fig. 1*A*). However, a decreased proline content in tobacco leaf discs was observed at Cu concentration higher than 0.1 mM (Fig. 3) and no wilting symptoms were shown.

In air-dried leaf discs, water deficit increased *P5CS* and *OAT* expression, but repressed *PDH*. Though 0.1 to 1 mM Cu treatments did not completely repress *PDH* expression, they also did not markedly stimulate the expression of the proline synthesis genes compared to control conditions. *PDH* expression was completely repressed by 5 mM Cu and under severe water stress, this Cu concentration failed to stimulate the expression of *P5SC* and *OAT*, in contrast to water stress (Fig. 2*B*). The water stress increased the expression of *P5CS* and *OAT* and repressed *PDH* expression within 6 h, whereas Cu treatment decreased the expression of genes only as treatment progressed.

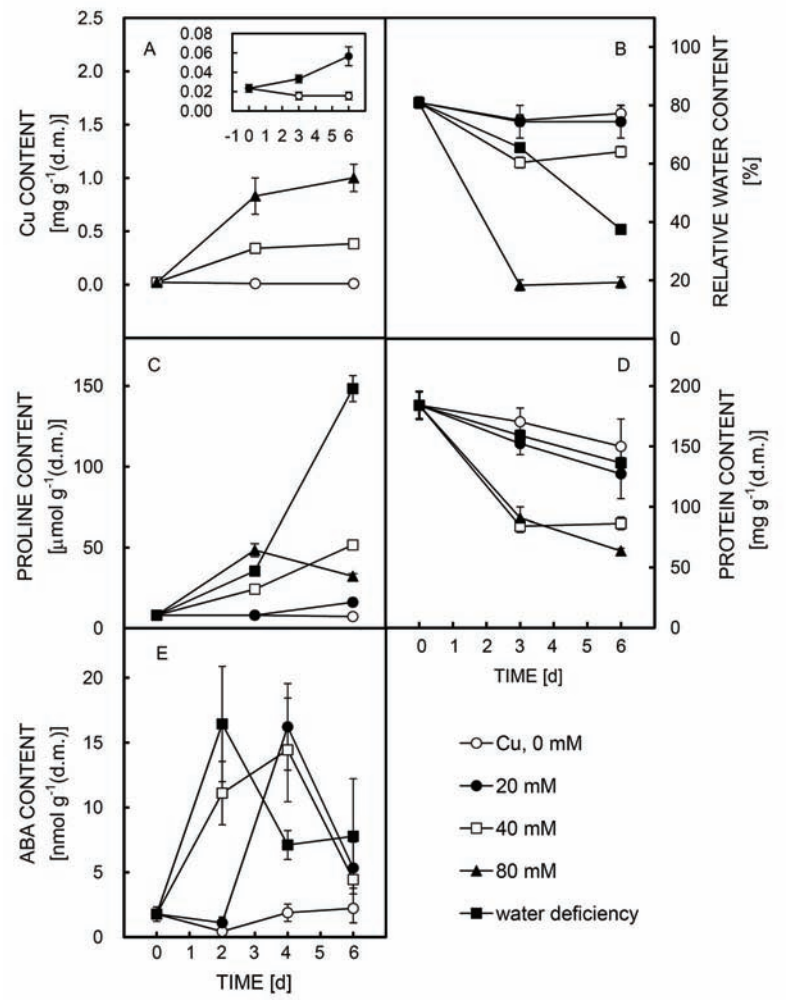

Fig. 1. Time course showing leaf Cu content (*A*), relative water content (*B*), proline content (*C*), protein content (*D*) and abscisic acid (ABA) content (*E*) of tobacco potted plants treated with different concentrations of $CuSO_4$ or water stress. The graph inside the upper left panel shows the leaf Cu content [mg g^{-1}(d.m.)] in leaves treated with 20 mM $CuSO_4$ for 0 to 6 h. Means ± SE, $n = 3$.

Discussion

This paper presents the expression of three proline metabolism related genes, *P5CS*, *OAT* and *PDH* and certain physiological evidences to distinguish the effects of water deficit and excess copper on proline metabolism in tobacco. We have cloned *Nicotiana benthamiana OAT*, *PDH* and *P5CS* gene fragments (Ku *et al.*, unpublished data) and they showed similar patterns as the cloned tomato gene fragments. Excess Cu treatments to potted plants increased proline content of tobacco leaves (Fig. 1*C*) accompanied by a decrease of relative water content (Fig. 1*B*) and an increase in Cu content (Fig. 1*A*), suggesting both responses might be involved in the regulation of proline accumulation. Though the proline content decreased at the 6th day of 80 mM Cu treatment, the Cu content in leaves reached more than 1 mg g^{-1}(d.m.) (Fig. 1*A*,*C*). This indicates that the tobacco leaves probably might not tolerate too high Cu content. In addition, the accumulation of proline was correlated with the decrease of RWC of tobacco leaves (Fig. 1*B*,*C*). The amount of proline induced by water deficit reached 150 µmol g^{-1}(d.m.), which was about 3 times higher than that induced by any of the Cu treatments (Fig. 1*C*).

In order to distinguish the participation of water deficit and excess copper on proline metabolism, tobacco leaf discs were floated on solutions with various Cu concentrations. Cu in all concentrations tested did not increase proline content and in contrast to potted plants proline content decreased at higher Cu concentrations. In addition, when the Cu content in intact leaves was above 1 mg g^{-1}(d.m.) (under 80 mM Cu treatment) the water deficit-induced proline accumulation ceased and the proline content even decreased (Fig 1*C*). Thus, it is

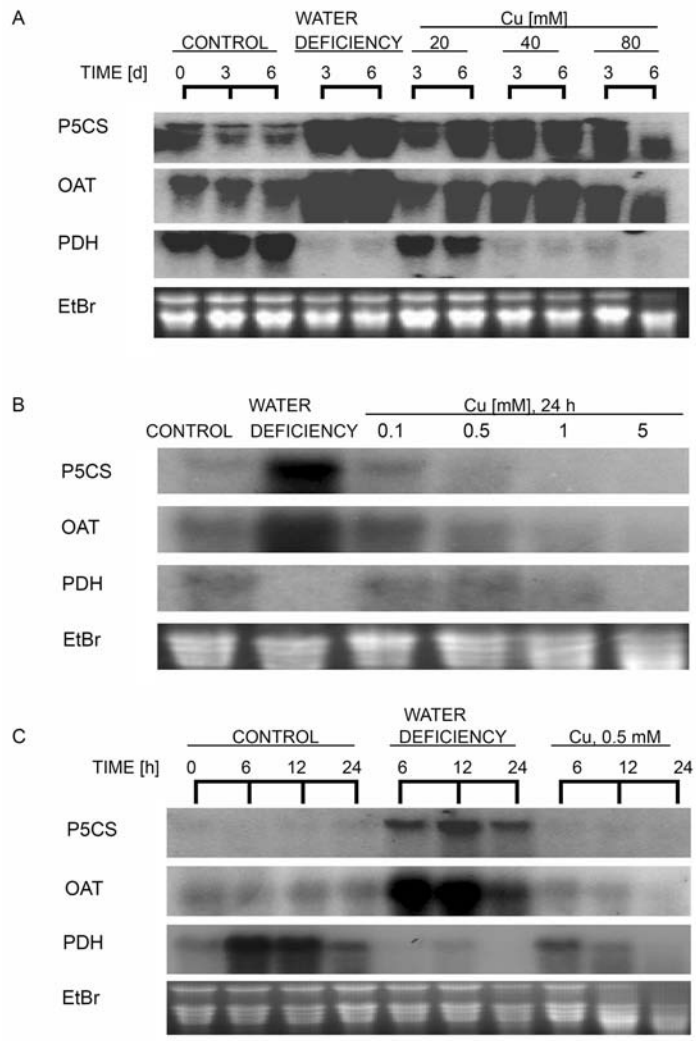

Fig. 2. RNA blot analysis of leaf *OAT*, *P5CS* and *PDH* gene expression of potted tobacco plants (*A*) or leaf discs (*B* and *C*) treated with different concentrations of $CuSO_4$ or water deficit. The bottom panel is the ethidium bromide-stained gel prior to RNA transfer.

possible that proline accumulation does not result from direct effect of Cu ions, but rather from Cu-induced water deficit.

Numerous studies have revealed that the reciprocal regulation of proline synthesis and catabolism plays a key role in the pattern of water, osmotic and salt stresses (Kiyosue *et al.* 1996, Peng *et al.* 1996, Verbruggen *et al*. 1996, Trotel-Aziz *et al.* 2000), but little is known about heavy metal stress. The mild Cu stress induced proline accumulation in tobacco leaves of potted plants along with the increased expression of two proline synthesis genes, *P5CS* and *OAT*, and decreased expression of proline catabolism gene, *PDH*. The similar gene expression pattern was confirmed in the water-deficient treatment. The decline of proline may have been caused by the inhibition of the expression of *P5CS* and *OAT* (Fig. 2*A*). The expression of *PDH* was inhibited by excess Cu ions in solution (Fig. 2*A*), suggesting that excess Cu ions inhibit both the synthesis and the catabolism of proline in floating disks (Fig. 3*B*,*C*).

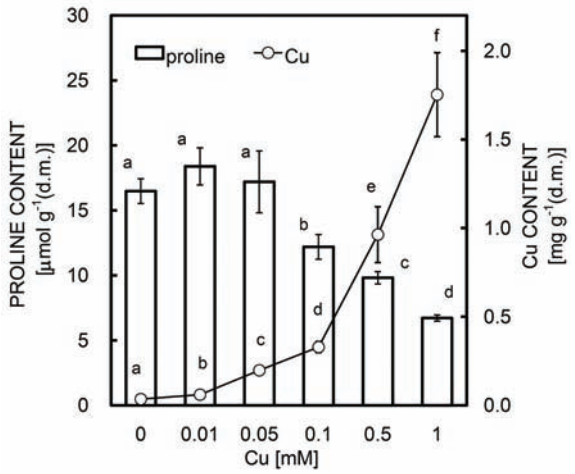

Fig. 3. Copper and proline content of tobacco leaf discs floated on different concentrations of $CuSO_4$ for 24 h. Means ± SE, $n = 3$. The means labeled with different letters show significant difference among treatments ($P < 0.05$).

Many studies have suggested that stress-induced proline accumulation could result from an increase in protein degradation (Charest and Phan 1990, Chen *et al.* 2001). Our results showed that water deficit-induced proline accumulation was not related to the turnover of protein. However, the 40 and 80 mM Cu treatments were associated with a marked decrease in protein content (Fig. 1*D*), in which the corresponding proline accumulation might result from a stimulation of protein catabolism.

Abscisic acid is thought to play an important role in proline accumulation (Chou *et al*. 1991, Pesci and Reggiani 1992, Savoure *et al.* 1997, Trotel-Aziz *et al.* 2000). Our results showed that both water deficit and Cu treatment induce an ABA increase in the early stage of the treatments (Fig. 1*E*). It is suggested that ABA-induced by excess Cu might play a signaling role for the initiation of gene regulation related to proline accumulation. Hsu and Kao (2003) found that the increase of ABA in rice content induced by cadmium reduced transpiration and thus led to decreased Cd transport to the shoot. Our results demonstrated that the amount of Cu ion transported to tobacco leaves was retarded after three days at 40 or 80 mM Cu, which occurred right after the increase of ABA. Thus, Cu treatment might reduce water transport in potted plants, which in turn induced water deficit in tobacco shoots.

The inhibitory effect of Cu ions on proline metabolism seems to reduce the protective role of proline and may reduce the longevity of plant. In addition, the increase of ABA induced by Cu may reduce water transport and down-regulate metal absorption efficiency. Most of polluted sites are contaminated with multiple heavy metals. Our unpublished results of floating tobacco leaf discs on the cadmium, chromium and zinc solutions show that, unlike Cu, these heavy metals can induce proline accumulation in tobacco leaf discs. This suggests that the existence of Cu in sites polluted with multiple heavy metals might enhance the toxicity of other heavy metals, because the coexisting Cu may inhibit the increase of proline that could be induced by other heavy metals.

References

Armengaud, P., Thiery, L., Buhot, N., March, G.G., Savoure, A.: Transcriptional regulation of proline biosynthesis in *Medicago truncatula* reveals developmental and environmental specific features. - Physiol. Plant. **120**: 442-450, 2004.

Bassi, R., Sharma, S.S.: Proline accumulation in wheat seedlings exposed to zinc and copper. - Phytochemistry **33**: 1339-1342, 1993.

Bates, L.S., Waldren, R.P., Teare, I.D.: Rapid determination of free proline for water-stress studies. - Plant Soil **39**: 205-207, 1973.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities for protein utilizing the principles of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Charest, C., Phan, C.T.: Cold accumulation of wheat (*Triticum aestvum*): properities of enzymes involved in proline metabolism. - Physiol. Plant. **80**: 159-168, 1990.

Canas, R.A., Villalobos, D.P., Diaz-Moreno, S.M., Canovas, F.M., Canton, F.R.: Molecular and functional analyses support a role of ornithine-δ-aminotransferase in the provision of glutamate for glutamine biosynthesis during pine germination. - Plant Physiol. **148**: 77-88, 2008.

Chen, C.T., Chen, L.M., Lin, C.C., Kao, C.H.: Regulation of proline accumulation in detached rice leaves exposed to excess copper. - Plant Sci. **160**: 283-290, 2001.

Chen, C.T., Chen, T.H., Lo, K.F., Chiu, C.Y.: Effects of proline on copper transport in rice seedlings under excess copper stress. - Plant Sci. **166**: 103-111, 2004.

Chen, C.T., Kao, C.H.: Osmotic stress and water stress have opposite effects of putrescine and proline production in excised rice leaves. - Plant Growth Regul. **13**: 197-202, 1993.

Chou, I.T., Chen, C.T., Kao, C.H.: Characteristics of the induction of the accumulation of proline by abscisic acid and isobutyric acid in detached rice leaves. - Plant Cell Physiol. **32**: 269-272, 1991.

Delauney, A.J., Verma, D.P.S.: Proline biosynthesis and osmoregulation in plants. - Plant J. **4**: 215-223, 1993.

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

Fujita, T., Maggio, A., Garcia-Rios, M., Bressan, R.A., Csonka, L.N.: Comparative analysis of regulation of expression and structures of two evolutionarily divergent genes for Δ^1-pyrroline-5-carboxylate synthetase from tomato. - Plant Physiol. **118**: 661-674, 1998.

Funck, D., Stadelhofer, B., Koch, W.: Ornithine-δ-aminotransferase is essential for arginine catabolism but not for proline biosynthesis. - BMC Plant Biol. **8**: 40, 2008.

Girousse, C., Bournocille, R., Bonnemain, J.L.: Water deficit-induced changes in concentrations in proline and some other amino acids in the phloem sap of alfalfa. - Plant Physiol. **111**: 109-113, 1996.

Haag-Kerwer, A., Schafer, H.J., Heiss, S., Walter, C., Rausch, T.: Cadmium exposure in *Brassica juncea* causes a decline in transpiration rate and leaf expansion without effect on photosynthesis. - J. exp. Bot. **341**: 1827-1835, 1999.

Hervieu, F., Le Dily, F., Huault, C., Billard, J.P.: Contribution of ornithine aminotransferase to proline accumulation in NaCl-treated radish cotyledons. - Plant Cell Environ. **18**: 205-210, 1995.

Hsu, Y.T., Kao, C.H.: Role of abscisic acid in cadmium tolerance of rice (*Oryza sativa* L.) seedlings. - Plant Cell Environ. **26**: 867-874, 2003.

Hu, C.A.A., Delauney, A.J., Verma, D.P.S.: A bifunctional enzyme (Δ^1-pyrroline- 5-carboxylate synthetase) catalyzes the first two steps in proline biosynthesis in plants. - Proc. nat. Acad. Sci. USA **89**: 9354-9358, 1992.

Jan, F.J., Pang, S.Z., Fagoaga, F., Gonsalves, D.: Turnip mosaic

potyvirus resistance in *Nicotiana benthamiana* derived by post-transcriptional gene silencing - Transgen. Res. **8**: 203-213, 1999.

Jan, F.J., Pang, S.Z., Tricoli, D.M., Gonsalves, D.: Evidences that plant developmental stage and combining transgene from different lines enhance resistance in squash mosaic comovirus coat protein transgenic plants - J. gen. Virol. **81**: 2299-2306, 2000.

Kandpal, R.P., Rao, N.A.: Water stress induced alterations in the properties of ornithine aminotransferase from ragi (*Eleusine coracana*) leaf enzymes - Biochem. Internat. **5**: 297-302, 1982.

Kastori, R., Petrovic, M., Petrovic, N.: Effects of excess lead, cadmium, copper, and zinc on water relations in sunflower - J. Plant Nutr. **15**: 2427-2439, 1992.

Kiyosue, T., Yoshiba, Y., Yamaguchi-Shinozaki, K., Shinozaki, K.: A nuclear gene encoding mitochondrial proline dehydrogenase, an enzyme involved in proline metabolism, is upregulated by proline but downregulated by dehydration in *Arabidopsis* - Plant Cell **8**: 1323-1335, 1996.

Mehta, S.K., Gaur, J.P.: Heavy-metal-induced proline accumulation and its role in ameliorating metal toxicity in *Chlorella vulgaris* - New Phytol. **143**: 253-259, 1999.

Nagoor, S.A., Vyas, A.V.: Physiological and biochemical responses of cereal seedlings to graded levels of heavy metals. III. Effects of copper on protein metabolism in wheat seedlings - J. environ. Biol. **20**: 125-129, 1999.

Napoli, C., Lemieux, C., Jorgensen, R.: Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. - Plant Cell **2**: 279-289, 1990.

Oncel, I., Keles, Y., Ustum, A.S.: Interactive effects of temperature and heavy metals stress on the growth and some biochemical compounds in wheat seedlings - Environ. Pollut. **107**: 315-320, 2000.

Pang, S.Z., Jan, F.J., Gonsalves, D.: Non-target DNA sequences reduce the transgene length necessary for RNA-mediated topovirus resistance in transgenic plants - Proc. nat. Acad. Sci USA **94**: 8261-8266, 1997.

Peng, Z., Lu, Q., Verma, D.P.S.: Reciprocal regulation of Δ^1-pyrroline-5-carboxylate synthetase and proline dehydrogenase genes controls proline levels during and after osmotic stress in plants - Mol. gen. Genet. **253**: 334-341, 1996.

Pesic, P., Reggiani, R.: The process of abscisic acid-induced proline accumulation and the levels of polyamines and quaternary ammonium compounds in hydrated barley leaves - Physiol. Plant. **84**: 134-139, 1992.

Roosens, N.H., Thu, T.T., Iskandar, H.M., Jacobs, M.: Isolation of the ornithine-δ- aminotransferase cDNA and effect of salt stress on its expression in *Arabidopsis thaliana* - Plant Physiol. **117**: 263-271, 1998.

Sambrook, J., Russell, D (ed.): Molecular Cloning: a Laboratory Manual. - Cold Spring Harbor Laboratory Press, Cold Spring Harbor - New York 2001.

Saradhi, A., Saradhi, P.P.: Proline accumulation under metal stress - J. Plant Physiol. **138**: 554-558, 1991.

Savoure, A., Hua, X.J., Bertauche, N., Montagu, M., Verbruggen, N.: Abscisic acid-independent and abscisic acid-dependent regulation of proline biosynthesis following cold and osmotic stresses in *Arabidopsis thaliana* - Mol. gen. Genet. **254**: 104-109, 1997.

Savoure, A., Jaoua, S., Hua, X.J., Ardiles, W., Van Montagu, M., Verbruggen, N.: Isolation, characterization, and chromosomal location of a gene encoding the Δ^1-pyrroline-5-carboxylate synthetase in *Arabidopsis thaliana* - Feder. Eur. Biochem. Soc. Lett. **372**: 13-19, 1995.

Schat, H., Sharma, S.S., Vooijs, R.: Heavy metal-induced accumulation of free proline in a metal-tolerant and a non-tolerant ecotype of *Silene vulgaris* - Physiol. Plant. **101**: 477-482, 1997.

Sharma, S.S., Dietz, K.J.: The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress - J. exp. Bot. **57**: 711-726, 2006.

Siripornadulsil, S., Traina, S., Verma, D.P.S., Sayre, R.T.: Molecular mechanisms of proline-mediated tolerance to toxic heavy metals in transgenic microalgae - Plant Cell **14**: 2837-2847, 2002.

Talanova, V.V., Totov, A.F., Boeva, N.P.: Effect of increasing concentrations of lead and cadmium on cucumber seedlings - Biol. Plant. **43**: 441-444, 2000.

Thippeswamy, M., Chandraobulreddy, P., Sinilal, B., Shiva Kumar, M., Chinta Sudhakar: Proline accumulation and the expression of Δ^1-pyrroline-5-carboxylate synthetase in two safflower cultivars - Biol. Plant. **54**: 386-390, 2010.

Thomas, J.C., Malick, F.K., Endreszl, C., Davies, E.C., Murray, K.S.: Distinct responses to copper stress in the halophyte *Mesembryanthemum crystallinum* - Physiol. Plant. **102**: 360-368, 1998.

Tripathi, A.K., Tripathi, S.: Changes in some physiological and biochemical characters in *Albizia lebbek* as bio-indicators of heavy metal toxicity - J. environ. Biol. **20**: 93-98, 1999.

Trotel-Aziz, P., Niogret, M.F., Larher, F.: Proline level is partly under the control of abscisic acid in canola leaf discs during recovery from hyper-osmotic stress - Physiol. Plant. **110**: 376-383, 2000.

Turchetto-Zolet, A.C., Margis-Pinheiro, M., Margis, R.: The evolution of pyrroline-5- carboxylate synthase in plants: a key enzyme in proline synthesis - Mol. gen. Genet. **281**: 87-97, 2009.

Verbruggen, N., Hua, X., May, M., Montagu, M.V.: Environmental and developmental signals modulated proline homeostasis: evidence for a negative transcriptional regulator - Proc. nat. Acad. Sci. USA **93**: 8787-8791, 1996.

Voetberg, G.S., Sharp, R.E.: Growth of the maize primary root at low water potentials. III. Role of increased proline deposition in osmotic adjustment. - Plant Physiol. **96**: 1125-1130, 1991.

Walker, D.J., Romero, P., Correal, E.: Cold tolerance, water relations and accumulation of osmolytes in *Bituminaria bituminosa*. - Biol. Plant. **54**: 293-298, 2010.

Wu, L., Fan, Z., Guo, L., Li, Y., Chen, Z.L., Qu, L.J.: Over-expression of the bacterial *nhaA* gene in rice enhances salt and drought tolerance. - Plant Sci. **168**: 297-302, 2005.

Yoshiba, Y., Kiyosue, T., Nakashima, K., Yamaguchi-Shinozaki, K., Shinozaki, K.: Regulation of levels of proline as an osmolyte in plants under water stress. - Plant Cell Physiol. **38**: 1095-1102, 1997.