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Full Length Research Paper

The effect of NaCl on proline metabolism in *Saussurea amara* seedlings

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Many plants accumulates proline dramatically under a variety of stress conditions, and this play an important role in regulation of osmotic homeostasis, prevention of damages caused by osmotic stresses, scavenging of reactive oxygen species and protection of cell structures. The regulation of proline accumulation in *Saussurea amara* seedlings and the activities of key enzymes involved in proline metabolism in response to salinity were studied, and 50-days-old seedlings were treated with NaCl (0, 100, 200, 300 and 400 mM) in this experiment. The results showed that proline contents were higher in leaves than in roots and increased significantly with NaCl concentration and treatment duration ($P < 0.05$). The activity of pyrroline-5-carboxylate synthetase (P5CS) increased at first and than decreased with increaso of NaCl concentration. The activities of orn-δ-aminotransferase (δ-OAT) and proline dehydrogenase (ProDH) increased and decreased, respectively, in response to elevated NaCl concentration and treatment duration. The data demonstrated that glutamate and ornthine pathway were activated by NaCl, and proline biosynthesis mainly depended on glutamate pathway under low-concentration NaCl condition, in which P5CS took dominant position. Conversely, the ornthine pathway was predominant.

Key words: *Saussurea amara* seedlings, salinity stress, proline metabolism.

INTRODUCTION

Salinity is one of the major abiotic stresses affecting plant growth, development and productivity. This constraint effects through osmotic inhibition, ionic toxicity and disturbance of the uptake and translocation of nutritional ions, disturb the physiological and biochemical functions of the plant cell and leads to cell death (Misra and Dwivedi, 2004; Xiong and Zhu, 2002). It is well known that praline (Pro) is a major organic molecule which accumulates in many plants exposed to environmental stresses such as drought and salinity (Nanjo et al., 1999; Kuznetsov and Shevyakova, 1997). The level of proline accumulation in plants varies from species to species and can be 100 times greater than in control situation (Nathalie and Christian, 2008). Proline accumulation may be a part of the stress signal influencing adaptive responses (Maggio et al., 2002).

Proline accumulation has been suggested to result from: (a) Decreased proline degradation, (b) increased proline biosynthesis, (c) lower proline utilization, and (d) protein degradation (Delauney and Verma, 1993). In higher plants, proline is synthesized via both glutamate (Glu) and ornithine (Orn) pathways. The glutamate pathway is catalyzed by a single bifunctional enzyme, pyrroline-5-carboxylate synthetase (P5CS) and produces gultamic-γ-semialdehyde (GSA), which is spontaneously converted to pyrroline-5-carboxylate (P5C) and then reduced to proline (Nathalie and Christian, 2008; Maurizio et al., 2008; Silke et al., 2010). Under many

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Abbreviations: Pro, Proline; **Glu,** glutamate; **Orn,** ornithine; **P5CS,** pyrroline-5-carboxylate synthetase; **GSA,** gultamic-γ-semialdehyde; **P5C,** pyrroline-5-carboxylate; **δ-OAT,** orn-δ-aminotransferase; **ProDH,** proline dehydrogenase; **EDTA,** ethylenediaminetetraacetic acid; **PVPP,** polyvinylpolypyrrolidone; **ROS,** reactive oxygen species.

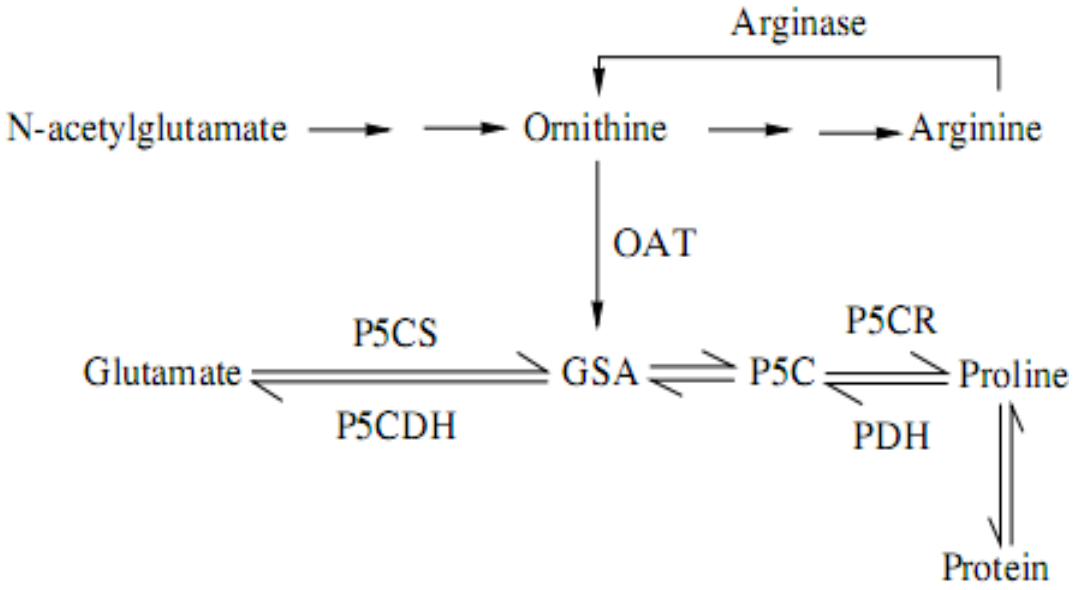

Figure 1. A schematic diagram of proline metabolism in plants (Song et al., 2005).

stress conditions, especially salinity and drought, proline accumulation is correlated with P5CS activity, as it was suggested to be the key regulatory and rate-limiting enzyme in the biosynthetic pathway. An alternative precursor for proline biosynthesis is Orn, which can be transaminated to P5C by orn-δ-aminotransferase (δ-OAT), a mitochondrial located enzyme. On the other hand, proline accumulation also depends on its degradation, which is catalyzed by the proline dehydrogenase (ProDH) and it is considered as another determinant enzyme in proline accumulation (Figure 1). It is proposed that a signalling cascade activated by stress-regulated changes in proline synthesis and/or degradation may modify the throughput of parallel cascades upstream of at least certain stress-inducible genes. This would provide a regulatory mechanism of continuously ensuring that the genetic response to stress is appropriate for the prevailing environmental conditions (Hare et al., 1999).

Saussurea amara, a perennial herb, is a typical halophyte living in saline soil and salinized meadow in north of China. It is a wild plant with high feeding value for its high protein content, vitamins and minerals. This plant plays an important role in keeping the ecological balance and molding landscape characteristic of saline and alkaline land (Song et al., 2002). These plants are exposed to several environmental stresses such as drought and salinity which could adversely affect plant growth and production. Therefore, it becomes necessary to elucidate the stress tolerance mechanisms.

Thus, the objective of this work was to investigate the effects of NaCl on *S. amara* of key enzymes involved in proline metabolism in order to clarify whether proline is of physiological significance for salt-tolerance acquisition in the halophyte plants.

MATERIALS AND METHODS

Plant material

Seeds of *S. amara* provided by Shanxi Agricultural University were sown and grown in a greenhouse under controlled environmental conditions, with relative humidity of 65 ~ 75 % and temperature 20 ~ 25℃. Five plants were grown in individual pots (25 cm diameter, 20 cm height) filled with complex substrate (sand : perlite, 3:1). The plants were irrigated weekly with Hoagland nutrient solution (Hoagland and Arnon, 1950). The NaCl treatment (0, 100, 200, 300 and 400 mM) was applied to 50-day-old-plants and plants were harvested at 0, 6, 12, 24, 48 and 72 h. Samples for proline and enzymatic assays were stored at -80℃.

Proline determination

Samples of 0.5 g were homogenized in 6 ml of 3% sulfosalicylic acid, and the homogenate was centrifuged at 3000 g for 20 min. Proline concentration was determined as described by Bates et al. (1973).

Enzyme assays

Frozen samples (approximately 5 g) were ground in liquid nitrogen and then extracted in 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM β-mercaptoethanol, 1% (w/v) polyvinylpolypyrrolidone (PVPP), 5 mM $MgCl_2$ and 0.6 M KCl. The homogenate was centrifuged at 12000 g for 20 min at 4℃ and the resulting supernatant was kept at -20℃ until enzyme assay. All operations were carried out at 4℃ (Lutts et al., 1999; Chen et al., 2001).

The P5CS activity was determined by monitoring the consumption of NADPH and measuring the increase in absorbance at 340 nm. Two milliliter reaction mixture containing 75 mM Glu, 100 mM Tris-HCl (pH 7.2), 20 mM $MgCl_2$, 5 mM ATP, 0.4 mM NADPH and 0.5 ml enzyme extract was incubated at 37℃ for 20 min, then the absorbance at 340 nm (Stines et al., 1999) was recorded. P5CS is expressed as unit per mg protein (one unit is defined as an increase in 0.001 A_{340} per min).

δ-OAT activity was assayed according to Vogel and Kopac (1960).

The assay mixture contained 0.2 ml enzyme extract and 0.8 ml of 100 mM potassium phosphate buffer (pH 8.0) containing 50 mM L-ornithine, 20 mM α-ketoglutarate and 1 mM pyridoxal-5'-phosphate. The reaction medium was incubated at 37°C for 30 min. The reaction was stopped by adding 0.5 ml trichloroacetic acid (10 %) and the colour was developed by incubating the reaction mixture with 0.5 ml o-aminobenzaldehyde (0.5 %, w/v, in ethanol) for 1 h. After centrifugation at 12000 g for 10 min, the clear supernatant fraction was collected to measure the absorbance at 440 nm. δ-OAT was expressed as unit per mg protein (one unit is defined as an increase in 0.001 A_{440} per min).

ProDH was assayed by directly measuring the NAD^+ reduction at 340 nm as described by Rena and Splittstosser (1975). Two milliliter of a 100 mM Sodium carbonate-bicarbonate buffer (pH 10.3) containing 20 mM L-proline, 10 mM NAD^+ and 0.5 ml enzyme extract was incubated at 25°C for 5 min, and the absorbance was measured at 340 nm. ProDH was expressed as unit per mg protein (one unit is defined as a decrease in 0.001 A_{340} per min).

Statistical analysis

All the presented data were mean values of at least three replications. The data were analysed statistically by using analysis of variance (ANOVA) technique. The treatments were compared by using the Least Significant Difference (LSD) test ($p \leq 0.05$).

RESULTS

Proline accumulation

Proline accumulation was affected by salt concentration and treatment duration and also by the interaction between these two factors. There was a gradual increase in proline concentration with the increase of treatment time. After 12 h, proline contents in salt-treated leaves increased significantly ($P < 0.05$) when compared to non-saline-treated leaves with the increase of treatment duration. The highest accumulation of proline in leaves was observed with 300 mM NaCl at 72 h (Figure 2A). Our results also demonstrated that proline content was higher in leaves when compared to roots and followed the same trend under 100 and 200 mM. Roots proline contents increased with salt treatment duration and reached the highest values at 200 mM NaCl (Figure 2B).

Enzymes of proline metabolism

Under many stress conditions, proline accumulation is correlated with P5CS, which is the key regulatory and rate-limiting enzyme in the biosynthetic pathway. P5CS catalyses the first step in the pathway of conversion of glutamate to proline. The result showed that the P5CS activity was higher in leaves by comparison with roots and that this activity increased at lower concentration of NaCl and then decreased with the rise of NaCl concentration. In the presence of 200 mM NaCl, the P5CS activity peaked in the leaves at 24 h, whereas in roots, the peak was observed at 72 h in the presence of 300 mM NaCl (Figure 3).

With regard to the metabolic pathway of ornithine, the NaCl treatments also significantly influenced the activity of δ-OAT. In our experiment, the activity of δ-OAT in the leaves increased with increasing NaCl concentration, presenting the highest activities at 72 h × 400 mM NaCl, with an increase of 4 times when compared with the control values (Figure 4A). The activity of δ-OAT in the roots changed in a similar way with some differences: it increased with the increase in NaCl concentration at 6, 12 and 24 h and it decreased with the highest concentration of NaCl (300 and 400 mM) at 48 and 72 h (Figure 4B).

The other major factor that controls proline levels in higher plants is its degradation. Proline is oxidized to P5C in plant mitochondria by ProDH (Rayapati and Stewart, 1991). Figure 5 shows the evolution of ProDH activity in control and treated plants. The activities of ProDH significantly decreased with increasing NaCl concentration and treatment duration in both leaves and roots ($P < 0.05$). Within the following 6 h, a rapid decrease of ProDH activity can be noticed at all NaCl concentrations. Until the end of the experiment, ProDH activity of the stressed plants remained significantly greater as compared to the control ($P < 0.05$).

DISCUSSION

For many years, proline has been known to be involved in the response to a number of environmental stresses, particularly salt and drought stress. Osmotic stresses are caused by excessive accumulation of salt in the soil, either directly, because of salinization, or indirectly, because of water loss. The decrease in soil water potential led to an alteration of the plant water status which may cause stomatal closure, photosynthesis reduction and thus growth inhibition. Another consequence is the production of reactive oxygen species (ROS) and the accumulation of toxic ions within the cell, causing severe damage to membrane structures, proteins, nucleic acids and lipids (Apel and Hirt, 2004). A response to osmotic stress widespread in plants consists of the accumulation of compatible osmolytes which are thought to protect cells against stress damage. Among these plant compatible osmolytes, proline is considered to be of major importance, as it has been reported to accumulate in a large number of species in response to stresses (Hare and Cress, 1997). Proline is a low molecular-weight osmoprotectant which helps to preserve structural integrity and cellular osmotic potential within different compartments of the cell (Iyer and Caplan, 1998). In the present study, there was a positive correlation between proline accumulation and treatment time in *S. amara*. However, the highest accumulation of proline was not observed at the highest concentration of NaCl, and the proline accumulation first increase, then tends to slowly decreased when the NaCl concentration is above a critical value. The results also demonstrated that the contents of proline in leaves were higher than that in roots ($P < 0.05$), which was different from place to place. Verbruggen et al., (1993) reported that levels of proline

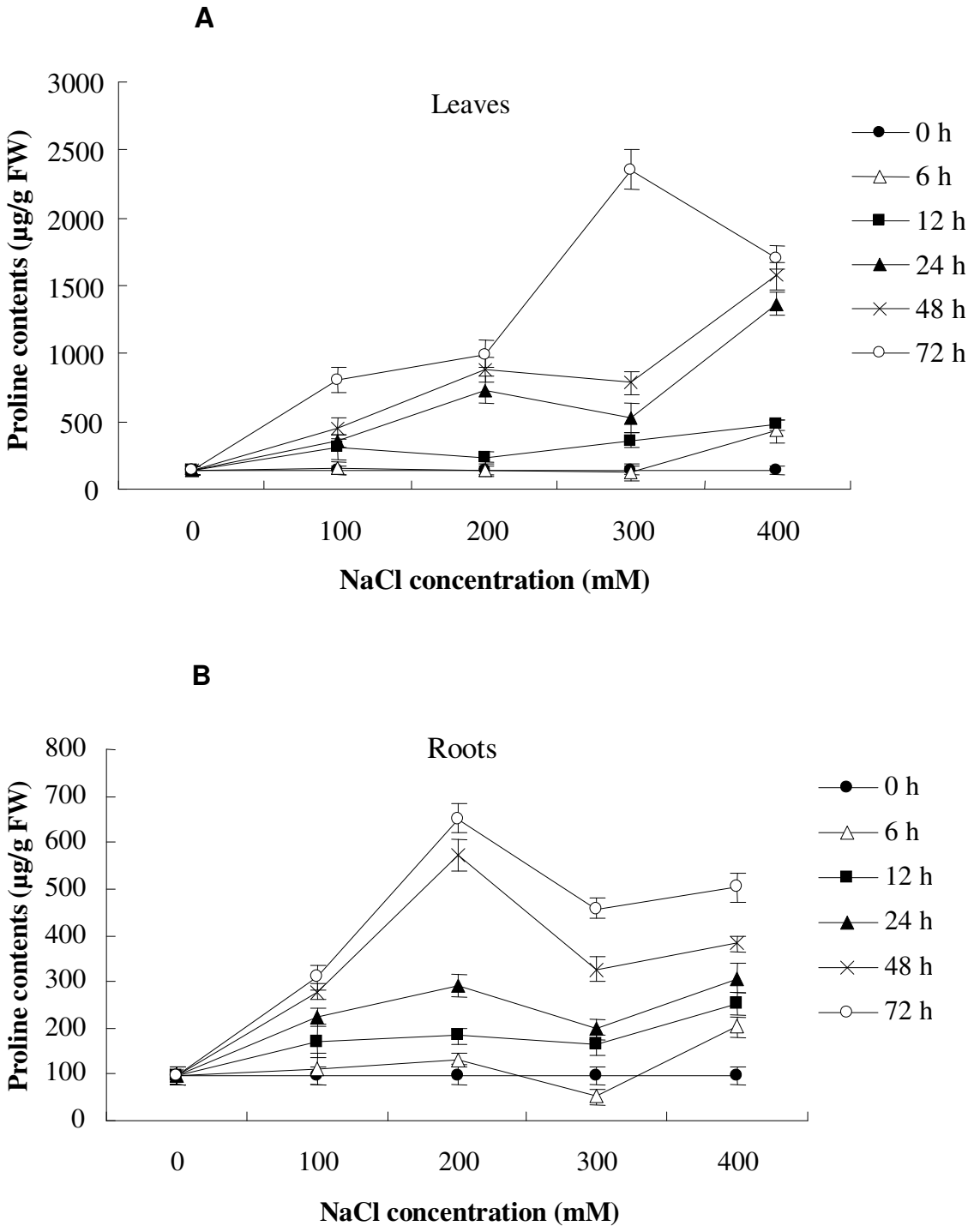

Figure 2. Effect of NaCl treatments on proline contents in *S. amara* seedlings (A: leaves, B: ROOTS). The values are means (±SE) of triplicate samples.

vary among plant organs, highest proline levels are found in flowers, especially in pollen grains, and in seeds, and lowest levels are found in roots. Proline accumulation usually focuses on the plant organs with high metabolism.

Proline metabolism is a typical biochemical adaptation in living organisms which is subjected to stress condition (Delauney and Verma, 1993). In maize seedlings, the treatments induced proline accumulation by activation of the biosynthetic pathway, including P5CS, δ-OAT and arginase (Yang et al., 2009). P5CS catalyses the first step in the pathway of conversion of glutamate to proline. During stress, the expression of P5CS is well correlated

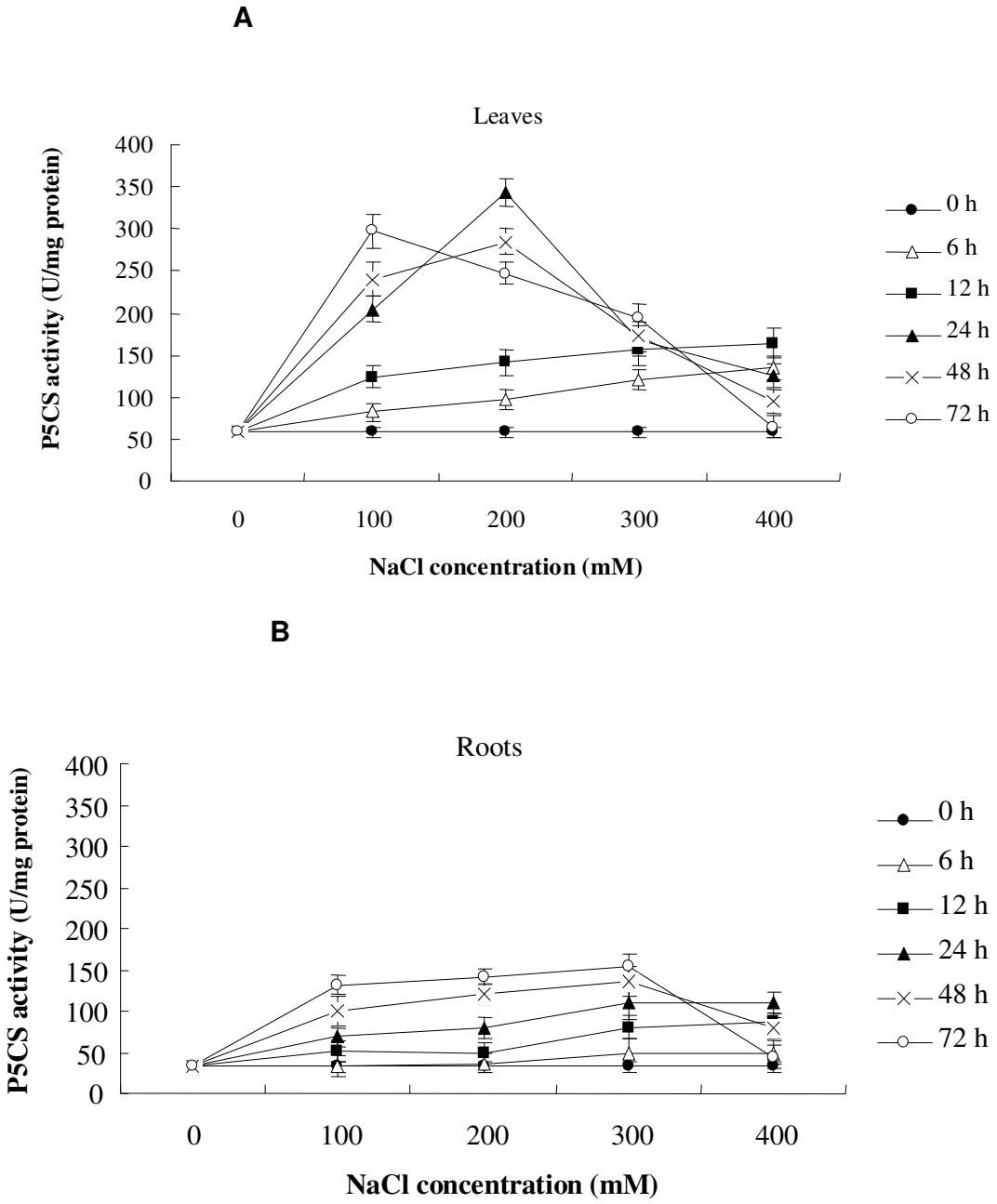

Figure 3. Effect of NaCl treatments on P5CS activity in *S. amara* seedlings (A: leaves, B: roots). The values are means (±SE) of triplicate samples.

with proline content (Yoshiba et al., 1995; Savoure et al., 1995). In *Medicago truncatula* (Armengaud et al., 2004) and in young *Arabidopsis* leaves (Roosens et al., 1998), induction of δ-OAT mRNA by osmotic stress has been reported, suggesting that both the glutamate and the ornithine pathways may contribute to proline accumulation under stress condition. In general, the effect of salt stress on the activities of enzymes was similar in the leaves and roots. In the experiment, the P5CS activity showed increasing pattern at low-concentration treatments (100 ~ 200 mM NaCl), and it decreased under high-concentration treatments (300 ~ 400 mM NaCl) (Figure 3). The δ-OAT activity increased during the whole experiment, and it increased rapidly in the leaves under high-concentration treatments (300 ~ 400 mM NaCl) (Figure 4). The results proved that in the proline biosynthesis, the glutamate pathway plays a leading role under low-concentration salt stresses, while the ornithine pathway plays a leading role under high-concentration salt stresses.

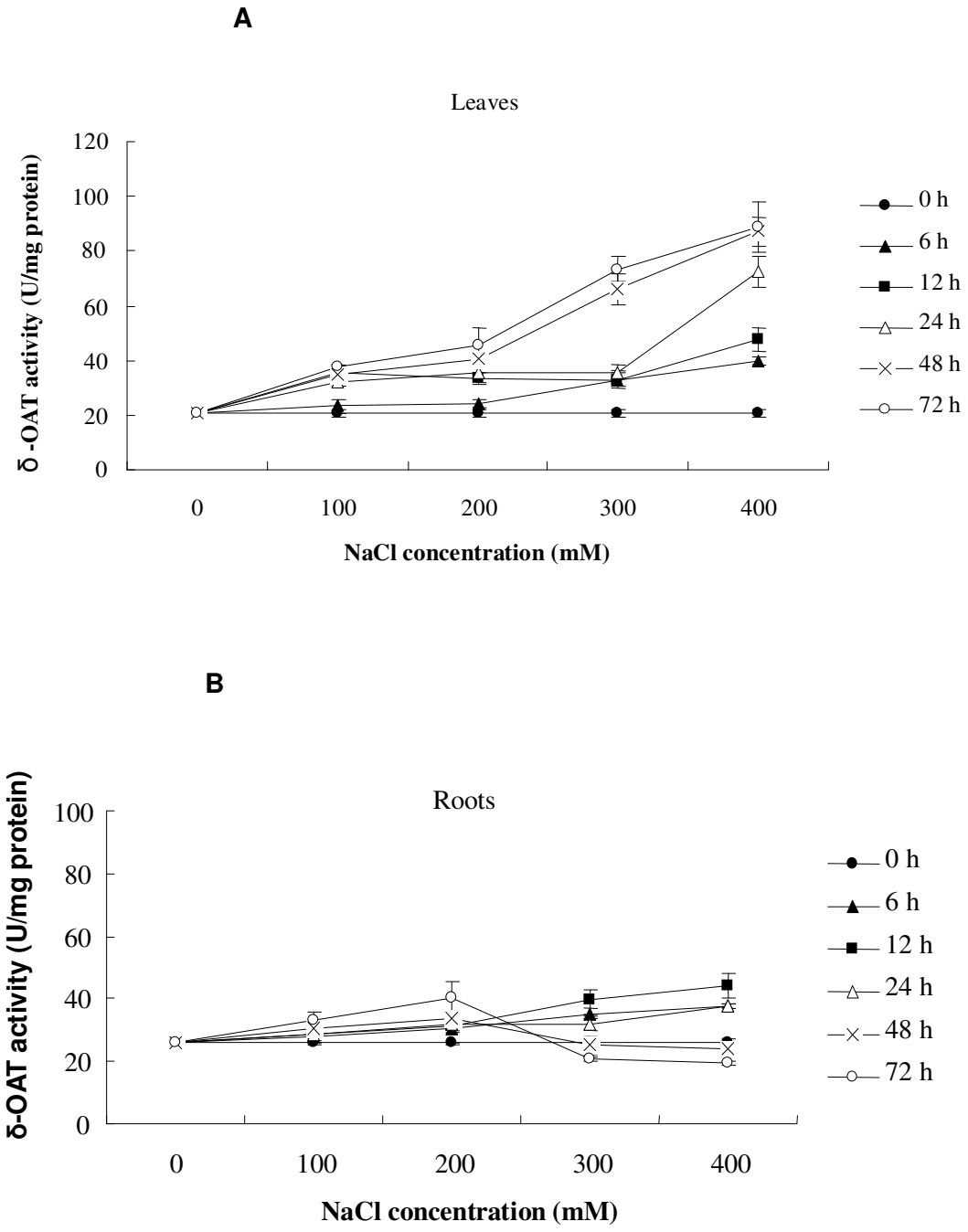

Figure 4. Effect of NaCl treatments on δ-OAT activity in *S. amara* seedlings (A: leaves, B: roots). The values are means (±SE) of triplicate samples.

Proline degradation is also regulated during development and stress. ProDH converts proline to glutamate. Thus, this enzyme also influences the level of proline. Rahnama and Ebrahimzadeh, (2004) reported that salt stress caused a reduction in the activities of ProDH in all the cultivars (*Solanum tuberosum*), and such reduction in ProDH activity and the simultaneous increase in proline level have also been reported in *Brassica juncea* under salt stress (Madan et al., 1995). Sumithra et al., (2006) reported that the activities of P5CS and δ-OAT significantly increased with progressive increase in salinity stress. In contrast, the activity of ProDH significantly decreased with increasing salt stress in both cultivars (*Vigna radiate*). In *Arabidopsis thaliana*, ProDH expression is low, except in flowers and young siliques, which also contain higher proline concentrations than in roots or

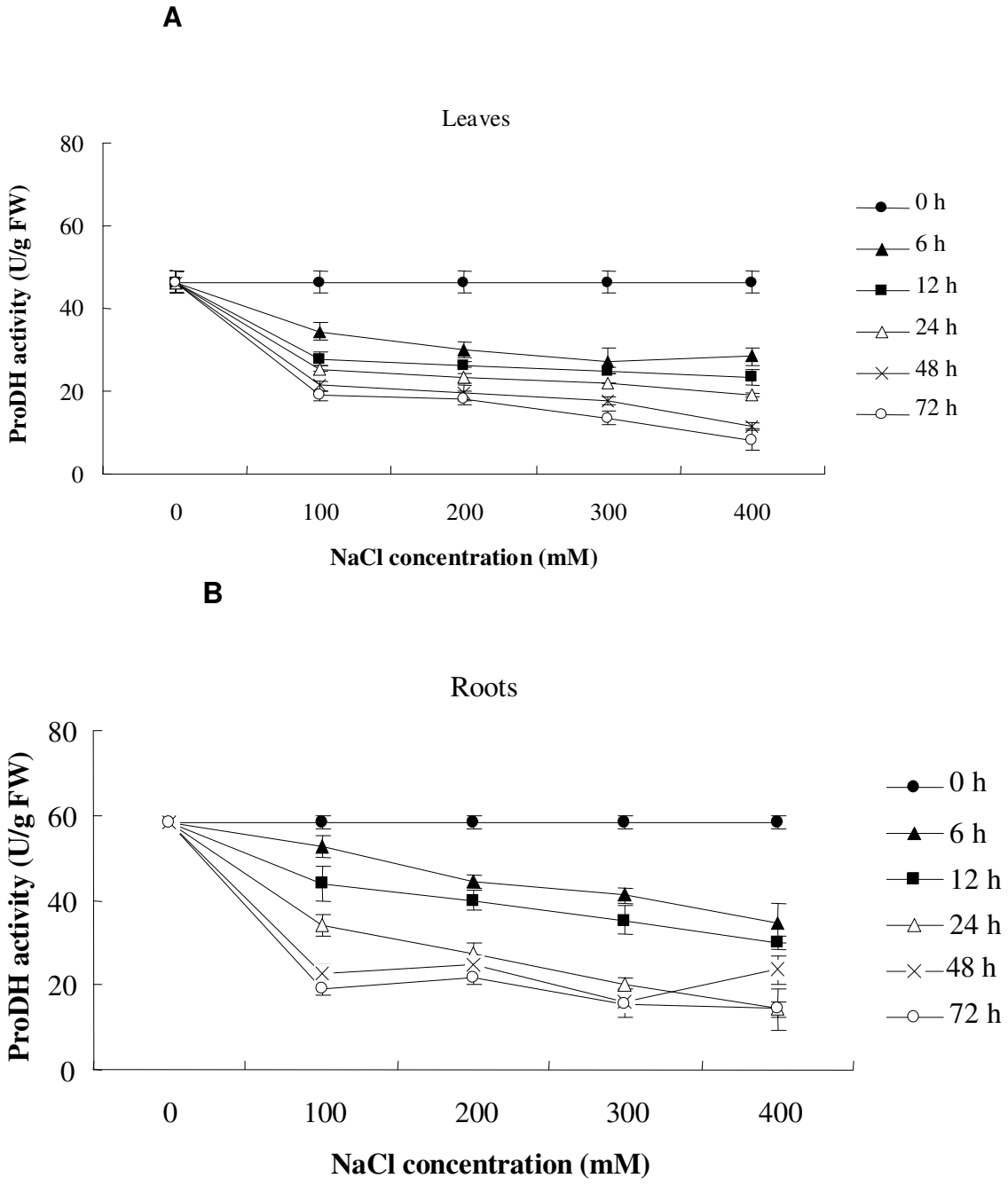

Figure 5. Effect of NaCl treatments on ProDH activity in *S. amara* seedlings (A: leaves, B: roots). The values are means (±SE) of triplicate samples.

leaves. ProDH expression is strongly induced by the addition of exogenously supplied proline. There is evidence for a negative transcriptional regulator which overrides the positive effect of accumulated proline on AtPDH expression (Verbruggen et al., 1996). It was proved by our results.

In conclusion, the stimulation of synthesis (increased P5CS and δ-OAT activities) in combination with an inhibition of oxidation (reduced ProDH activity) are the factors contributing to salinity stress induced free proline accumulation in *S. amara* seedlings. The enhanced activity of δ-OAT in the NaCl treatments provides an evidence for the participation of ornithine pathway in accumulating additional proline under salt stress, and the ornithine pathway plays a lead role under high-concentration salt stresses. The physiological significance of proline accumulation in ripened plants is not fully understood, and a clear relationship was not observed between

accumulation of proline and salt tolerance. Studying the effect of NaCl stress on enzyme activities involved in proline metabolism in *S. amara* seedlings could provide valuable information on the physiological significance of its accumulation. Regulation of proline accumulation in plants under NaCl stress is not similar to that under salt stress. However, the results provide some basic information which should be valuable for future studies.

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