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ORIGINAL PAPER

Signal regulation of proline metabolism in callus of the halophyte *Nitraria tangutorum* Bobr. grown under salinity stress

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Abstract Callus of the halophyte *Nitraria tangutorum* Bobr. was used to investigate proline metabolism and its signal regulation under salinity stress. Enhanced levels of proline and hydrogen peroxide (H_2O_2) were observed in calli exposed to salinity stress, and elevated levels of calcium (Ca) were detected in early responses to 75 mM NaCl treatment. Additionally, NaCl treatment induced significant elevation of ornithine-δ-aminotransferase (OAT) activity, but notable decreases occurred in the activities of glutamyl kinase (GK) and proline dehydrogenase (PDH). H_2O_2 scavenger dimethylthiourea and pyruvate inhibited the accumulation of proline and the stimulation of OAT in salinity-stressed calli. Moreover, the utilization of Ca chelator EGTA and Ca channel blocker verapamil abolished the enhancement of proline level induced by 75 mM NaCl treatment for 3 days. These results suggest that the accumulation of proline is correlated to the increase of OAT activity and the decrease of PDH activity in response to salinity, and that elevated Ca signal during the early stage of NaCl treatment and the excitation of OAT activity resulting from the increase of H_2O_2 generation are essential for proline accumulation in salinity-stressed calli.

Keywords Calcium · Hydrogen peroxide · *Nitraria tangutorum* Bobr. · Proline · Salinity

Abbreviations

ABA	Abscisic acid
Ca	Calcium
DMTU	Dimethylthiourea
GK	Glutamyl kinase
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol bis-(beta-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid
H_2O_2	Hydrogen peroxide
MS	Murashige and Skoog
OAT	Ornithine δ-aminotransferase
PDH	Proline dehydrogenase

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Introduction

High salt concentration in soils is one of the factors responsible for the decreases in plant growth and crop productivity in many regions of the world (Perez-Alfocea et al. 1996). Accumulation of proline is widely observed when plants are exposed to salt stresses (Kavi Kishor et al. 2005; Banu et al. 2009; Wang et al. 2011). Some researchers suggest that the elevation of proline level is merely due to stress reaction but not a mechanism for the increase of salt tolerance (Widholm 1988; Ashraf 1989). This idea is further supported by accumulating higher concentration of proline in sensitive rice cultivars than in tolerant genotypes under salt stress (Lutts et al. 1999). However, significant roles of proline involved in salt adaptation or salt tolerance have been demonstrated in some plants and cell cultures (Banu et al. 2009; Lokhande et al. 2010; Sekmen et al. 2012). A series of experiments provide the important evidence that the accumulation of proline can be used as a biochemical marker for the increase of salt tolerance in transgenic plants (Gleeson et al. 2005; Chen et al. 2007). For example, the study of Du et al. (2012) showed that transgenic plants of *Populus tomentosa* adapted to high salt stress environment and

accumulated proline, and that the overall salt tolerance was improved considerably.

Proline accumulation can be due to an increase in proline biosynthesis, via two different pathways from either glutamate or ornithine/ariginine (Kavi Kishor et al. 2005), and a decrease in proline degradation. Glutamyl kinase (GK) is a rate-limiting step in the glutamate pathway, and ornithine δ-aminotransferase (OAT) is a key enzyme which catalyses the first step in the ornithine pathway (Yang et al. 2009). In general, proline accumulation is associated with increased synthesis from glutamate (Misra and Gupta 2005). On the other hand, other investigations have suggested that the ornithine pathway, via an increase of OAT activity, contributes to proline synthesis when plants are treated with salinity (Madan et al. 1995; Krell et al. 2007). In addition, proline degradation catalysed by proline dehydrogenase (PDH) is involved in the accumulation of proline in response to salinity stress. For example, in *Saussurea amara* seedlings, salinity-induced proline accumulation is accompanied by a decrease in the activity of PDH (Wang et al. 2011).

Although previous studies have demonstrated the importance of proline accumulation in the adaptive response of plants to osmotic stress (Ashour and Mekki 2006; Megdiche et al. 2007), molecular signals involved in the regulation of proline metabolism are still poorly understood. An investigation on the role of abscisic acid (ABA) in the regulation of proline synthesis suggests that endogenous ABA content may affect proline accumulation in *Arabidopsis thaliana* under salt stress (Savoure et al. 1997). Calcium (Ca) plays a role as a signaling molecule in a number of plant responses to environmental stresses. By identifying the potential components of the signaling pathways required for the regulation of proline accumulation, Parre et al. (2007) suggested that Ca signal via phospholipase C is essential for proline accumulation under ionic hyperosmotic stresses in *Arabidopsis*. However, phospholipase D is a negative regulator of proline biosynthesis in *Arabidopsis thaliana* (Thiery et al. 2004). Hydrogen peroxide (H_2O_2) is also considered as a signal molecule regulating many important plant processes to different stresses including salinity. In a recent study, exogenous H_2O_2 treatment leads to a significant accumulation of proline in coleoptiles and radicles of maize seedlings (Yang et al. 2009), implying that H_2O_2 may participate in signal transduction events involved in the regulation of proline metabolism. These studies provide the evidence that various signals are responsible for regulating proline metabolism in response to different environmental stresses in some plants, whereas the causal link between H_2O_2 production and the changes of Ca content involved in the regulation of proline accumulation has seldom been studied in plants exposed to salinity stress.

Nitraria tangutorum Bobr. is a typical desert halophyte growing under extreme conditions in northwest China, which plays an important ecological function because of its superior tolerance to severe drought and high salinity. However, there are few researches on physiological adaptative responses of *Nitraria tangutorum* Bobr. to salt environment. More recently, our studies indicated that calli from *Nitraria tangutorum* Bobr. are an ideal material for studies on the adaptation of plants to salinity stress (Yang et al. 2010a) and that the higher activity of antioxidant enzymes plays an important role in salt tolerance of *Nitraria Tangutorum* Bobr. calli (Yang et al. 2010b). In this study, we used callus of *Nitraria tanguto*rum Bobr. to investigate proline metabolism and its signal regulation in response to salinity stress.

Materials and methods

Plant material and growth conditions

Seeds of *Nitraria tangutorum* Bobr. were obtained from Minqin Desert Botanical Garden of Gansu Province. The seeds were surface sterilized for 12 s with 75 % (v/v) ethanol, and then in 0.1 % (w/v) $HgCl_2$ for 10 min, the seeds were rinsed for 6 times with sterile distilled water. The embryos were extracted and incubated on 30 ml of growth regulator-free Murashige and Skoog (MS) solid medium. Callus was induced and subcultured as described in detail previously by Yang et al. (2010b).

NaCl (75, 150 and 300 mM), dimethylthiourea (DMTU, 5 mM), pyruvates (5 mM), ethylene glycol bis-(beta-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA, 4 mM) and verapamil (10 μM) were added in the MS medium. Calli were maintained at 24 °C ± 1.5 °C under a light irradiance of 150 μmol m^{-2} s^{-1} (10 h-photo period) for different days according to the experiment, then collected and washed by distilled water, the excess water was blotted with filter paper.

Proline measurement

After 3, 6 and 9 days of culture on MS medium containing 0.3 mg l^{-1} 6-BA, 1 mg l^{-1} NAA and different NaCl concentrations (0, 75, 150 or 300 mM), calli were collected for the measurement of proline content according to Bates et al. (1973) with some modifications. Fresh calli (0.5 g) were immediately homogenized in 5 ml of 3 % sulfosalicylic acid solution, and then heated at 98 °C for 10 min. After centrifugation at 15,000*g* for 15 min, 0.25 ml supernatant was added to 3.75 ml of the reaction medium containing 0.56 % sulfosalicyclic acid, 0.25 % glacial acetic acid and 1.25 % ninhydrin solution. The mixture was kept at 95 °C for 60 min, and then the reaction was

stopped quickly by an ice bath. Toluene (4 ml) was added to the mixture, and the organic phase was extracted and monitored at 520 nm by spectrophotometer.

Proline metabolism enzyme activity measurement

Calli cultured for 3, 6 and 9 days on MS medium containing 0.3 mg l^{-1} 6-BA, 1 mg l^{-1} NAA and different NaCl concentrations (0, 75, 150 or 300 mM) were collected in order to analyze OAT (EC 2.6.1.13), GK (EC 2.7.2.11) and PDH (EC 1.5.99.8) activities.

About 1 g of calli was immediately homogenized in 100 mM potassium phosphate buffer (pH 7.9) containing 1 mM ethylenediamine tetraacetic acid (EDTA), 15 % glycerol and 10 mM 2-mercaptoethanol. The homogenate was centrifuged at 15,000*g* for 15 min at 4 °C and the supernatant was collected for OAT activity measurement. OAT activity was assayed with ninhydrin according to Kim et al. (1994). One ml of the reaction mixture composed 50 mM Tris–HCl (pH 8.0), 50 mM L-ornithine, 5 mM β-ketoglutarate, 0.05 mM pyridoxal phosphate and the appropriate amount of crude enzyme extraction was incubated at 37 °C for 20 min. After adding 0.3 ml of 3 N perchloric acid and 0.2 ml of 2 % ninhydrin, the reaction was stopped by boiling for 5 min. The precipitate was collected by centrifugation (13,000*g*, 30 min, 4 °C) and completely dissolved with 1.5 ml of ethanol, and then the absorbance was recorded at 510 nm. The absorbance of 0.01 at 510 nm was defined as one unit (U) of OAT activity, and the specific enzyme activity was expressed as U mg^{-1} protein.

About 2 g of calli was ground in 2 ml TD buffer (50 mM Tris–HCl buffer, pH 7.0, 1 mM dithiothreitol and 10 % glycerol). After centrifugation at 16,000*g* for 20 min, the supernatant was collected and precipitated by adding solid ammonium sulphate (40 % saturation). And then, the soluble fraction obtained by centrifugation (16,000*g* for 20 min) was saturated with dry ammonium sulphate to a concentration of 80 %. After centrifugation at 15,000*g* for 15 min at 4 °C, the pellet was collected and completely dissolved with 1 ml TD buffer. The crude enzyme solution was obtained after a 24 h dialysis against TD buffer at 4 °C and GK activity was assayed by the method of Smith et al. (1984) with some modifications. A total volume of 1 ml assay mixture containing 50 mM glutamate, 10 mM ATP, 20 mM $MgCl_2$, 100 mM oxammonium hydrochloride, 50 mM Tris–HCl buffer (pH 7.0) and an appropriate amount of enzyme was incubated at 37 °C for 30 min and then the reaction was stopped by adding 1 ml stop solution (5.5 % $FeCl_3$, 2.0 % $HClO_4$, 2 M HCl). The precipitate was removed by centrifugation, and the absorbance of the supernatant at 535 nm was recorded against a blank identical to the one mentioned above but lacking ATP. The changes of absorbance of 0.01 h^{-1} at 535 nm were defined as one unit (U) of GK activity, and the specific enzyme activity was expressed as U mg^{-1} protein.

Fresh calli (0.5 g) were homogenized in the ice-cold extraction buffer (100 mM sodium phosphate, 1 mM cysteine and 0.1 mM EDTA, pH 8.0). After centrifugation at 15,000*g* for 10 min at 4 °C, the supernatant was used as crude enzyme preparation for the measurement of PDH activity. PDH activity was measured as described by Rena and Splittstoesser (1975) with a slight modification. In brief, the crude extraction was incubated in the reaction buffer (100 mM Na_2CO_3-$NaHCO_3$, 10 mM nicotinamide adenine dinucleotide (NAD), 20 mM L-proline, pH 10.3) at 32 °C for 5 min, and then PDH dependent NAD reduction was monitored at 340 nm for 4 min. One unit (U) of PDH activity was defined as an absorbance change of 0.001 min^{-1} and the specific enzyme activity was expressed as U mg^{-1} protein.

H_2O_2 content measurement

After 6 days of culture on MS medium containing 0.3 mg l^{-1} 6-BA, 1 mg l^{-1} NAA and various NaCl concentrations (0, 75, 150 and 300 mM), calli were collected for the measurement of H_2O_2 level according to the method of Sergiev et al. (1997) as described in detail previously by Yang et al. (2010b).

Determination of calcium content

Calli cultured for 0.25, 0.5, 1, 3 and 6 days on MS medium containing 0.3 mg l^{-1} 6-BA, 1 mg l^{-1} NAA and different NaCl concentrations (0, 75, 150 or 300 mM) were collected for the measurement of Ca content. Element ratio measurement was performed using a scanning electron microscope (Philips Electronics N.V., Eindhalen, the Netherlands) fitted with a Kevex energydispersive X-ray detector (Kenex, Valencia, CA, USA) as described by Vázquez et al. (1999) with some modifications. Calli were placed directly on the aluminum stage and quickly frozen under vacuum. The examination time of each sample was less than 10 min to avoid cell distortion. At least four to five cells per sample were examined. The results were calculated and the atomic number for a particular element in a given cell was expressed as a percentage of the total atomic number for all the elements measured (K, Na, Ca, Mg, P, S and Cl) in the cell.

After treatment with 0, 75 and 150 mM NaCl for 1 day, calli were collected for Ca^{2+} measurements according to Wu et al. (2002) with some modifications. Calli (15–20 g dry weight) were thoroughly washed with deionized water and dried at 60 °C for 3 days. The dry sample was extracted with 3 N HCl and ion content was determined

with an Inductive Coupled Plasma Emission Spectrometer (ICP, LABTAM8410).

Soluble protein content determination

The amount of soluble proteins was estimated according to the method of Bradford (1976) using bovine serum albumin as a standard.

Statistical analysis

Each experiment was repeated at least three times. Values were expressed as mean ± standard error (SE). Statistical comparisons were carried out using SPSS 13.0 software, and the treatment means were compared by using Duncan's multiple range test (DMRT) at $p < 0.05$.

Results

Effect of NaCl on proline content

Figure 1a shows the effect of NaCl on the level of proline in calli of *Nitraria tangutorum* Bobr.. During the early period of salt treatment, proline content increased significantly with the increase of NaCl concentrations and the maximal proline level reached 242 % of the control in calli after treatment with 300 mM NaCl for 3 days. However, proline content decreased with the duration of NaCl treatment. For example, there was about 44 % enhancement in the level of proline after 75 mM NaCl treatment for 3 days, but at the end of 75 mM NaCl treatment (9 days), no significant difference in proline content was observed, as compared with the control. Under 300 mM NaCl stress for 3, 6 and 9 days, the amount of proline was about 242, 172 and 156 % of the control, respectively.

Effects of NaCl on OAT, GK and PDH activities

The treatment of *Nitraria tangutorum* Bobr. calli with salinity led to a notable elevation of OAT activity (Fig. 1b). During the whole stress period, the maximal OAT activity was observed after treatment with NaCl for 6 days, while the increase rate of this enzyme activity decreased with prolonging stress time. Under 75 mM NaCl stress, OAT activity was 1.92, 2.04 and 1.23 folds of the control values in calli cultured for 3, 6 and 9 days, respectively. Further elevation of OAT activity was observed at 3, 6 and 9 days of 150 mM NaCl treatment, with approximately 2.45-, 2.73- and 1.92-fold increases as compared with the control, respectively.

In contrast to OAT, the treatment of NaCl induced significant decrease in GK activity (Fig. 1c). After exposuring calli to 75 mM NaCl for 3, 6 and 9 days, we found about 27, 36 and 38 % decrease in GK activity as compared with the control, respectively. A further decrease in GK activity was detected when calli were stressed with 150 mM NaCl.

Figure 1d shows the changes of PDH activity during the whole stress period. After 3 days of 75 and 150 mM NaCl treatment, PDH activity decreased about 24 and 31 % in comparison with the control, respectively, while 22 and 19 % reduction of this enzyme activity was induced in calli with prolonging NaCl treatment (6 days). After treatment with 75 and 150 mM NaCl for 9 days, PDH activity increased about 16 and 7 % of the control values, respectively (Fig. 1d), and the slight increase under only 75 mM NaCl treatment showed a significant difference in comparison with the unstressed level ($p < 0.05$).

NaCl-induced H_2O_2 production might be involved in the regulation of proline accumulation

The effect of 75 mM NaCl treatment on H_2O_2 production is shown in Fig. 2a. H_2O_2 concentration significantly increased in *Nitraria tangutorum* Bobr. calli with the duration of NaCl treatment, and a maximal increase was detected in NaCl-6 day-treated calli, with about 51 % elevation in comparison with control calli. Application of H_2O_2 scavengers DMTU and pyruvate could significantly reduce the increase of H_2O_2 content in salt-treated calli (Fig. 2b).

To confirm the relationship between H_2O_2 production and proline accumulation, the changes of proline content were measured in calli treated with 75 mM and 150 mM NaCl in the presence of H_2O_2 scavenger DMTU. Compared to the control, the treatment of 75 mM NaCl for 3 and 6 days induced a notable increase in proline content, but no significant difference in proline content after treatment of the calli with 75 mM NaCl was observed in the presence of 5 mM DMTU. The similar results were found in calli exposed to 150 mM NaCl treatment in the absence and in the presence of 5 mM DMTU (Fig. 3a). The application of pyruvate also reversed NaCl-induced increase of proline level when calli were exposed to 75 mM and 150 mM NaCl for 6 days (Fig. 3b).

Since the stimulation of OAT and the inhibition of PDH in response to salinity might be responsible for proline accumulation, we further investigated the effects of DMTU and pyruvate on the activities of these enzymes in salinity-treated calli. As shown in Fig. 4a, the increase of OAT activity was partly blocked by the addition of 5 mM DMTU or pyruvate in calli treated with 75 mM NaCl for 6 days, as compared with salinity treatment alone. However, the presence of DMTU and pyruvate did not show significant effects on salinity-induced decrease of PDH activity (Fig. 4b).

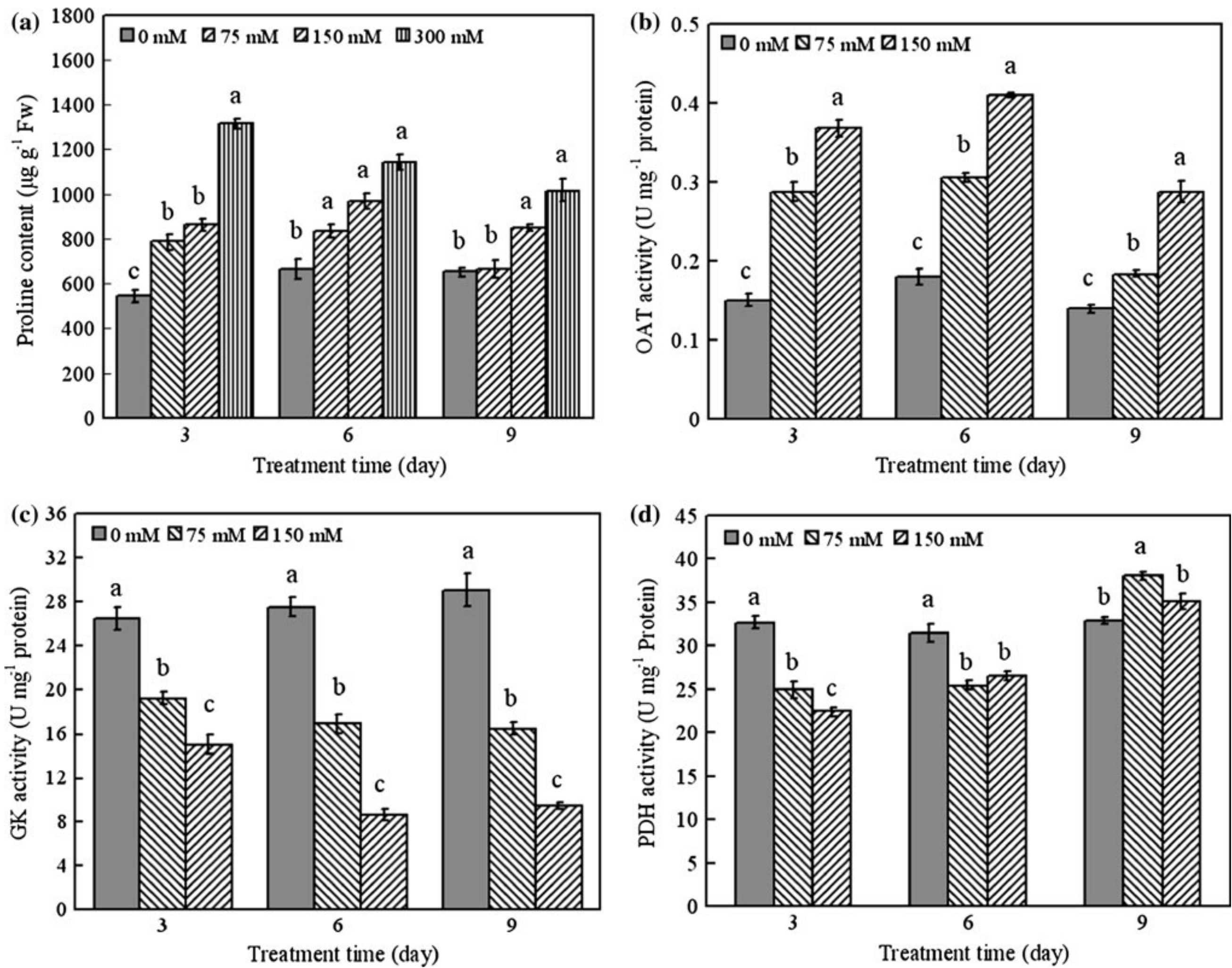

Fig. 1 Changes of proline content (**a**), and ornithine-δ-aminotransferase (OAT, **b**), glutamyl kinase (GK, **c**) and proline dehydrogenase (PDH, **d**) activities induced by NaCl treatment in calli of *Nitraria tangutorum* Bobr.. Data represent mean $\pm$ SE of at least three independent measurements. Within each day, *different letters* indicate significant differences at $p < 0.05$

Effect of NaCl on calcium content

As revealed in Fig. 5a, the amount of Ca was about 14.94, 21.65 and 21.63 % in control calli cultured for 1, 3 and 6 days, respectively, suggesting that Ca content increased with prolonging the culture. In comparison with the control, Ca percentage in calli increased about 49, 35 and 37 % after 75 mM NaCl treatment for 6, 12 and 24 h, respectively. However, after 3 and 6 days of NaCl treatment, about 24 and 30 % decrease of Ca percentage was induced as compared with the control, respectively. Likewise, measurement of Ca^{2+} content using an Inductive Coupled Plasma Emission Spectrometer showed that after 1 day of culture NaCl-treated calli accumulated more Ca^{2+} content than the untreated calli (Fig. 5b).

NaCl-induced calcium signal might be associated with proline accumulation

Further work was required to understand the relationship between Ca and NaCl-induced proline accumulation. As shown in Fig. 6, the addition of 4 mM EGTA abolished the enhancement of the amount of proline induced by NaCl treatment for 3 days, suggesting that Ca might be necessary to regulate proline accumulation in *Nitraria tangutorum* Bobr. calli when exposed to salinity. Additionally, when calli were treated with NaCl together with Ca channel blocker verapamil, NaCl-induced elevation in proline level was also reversed (Fig. 6), further demonstrating that Ca signal was responsible for proline accumulation in response to salinity.

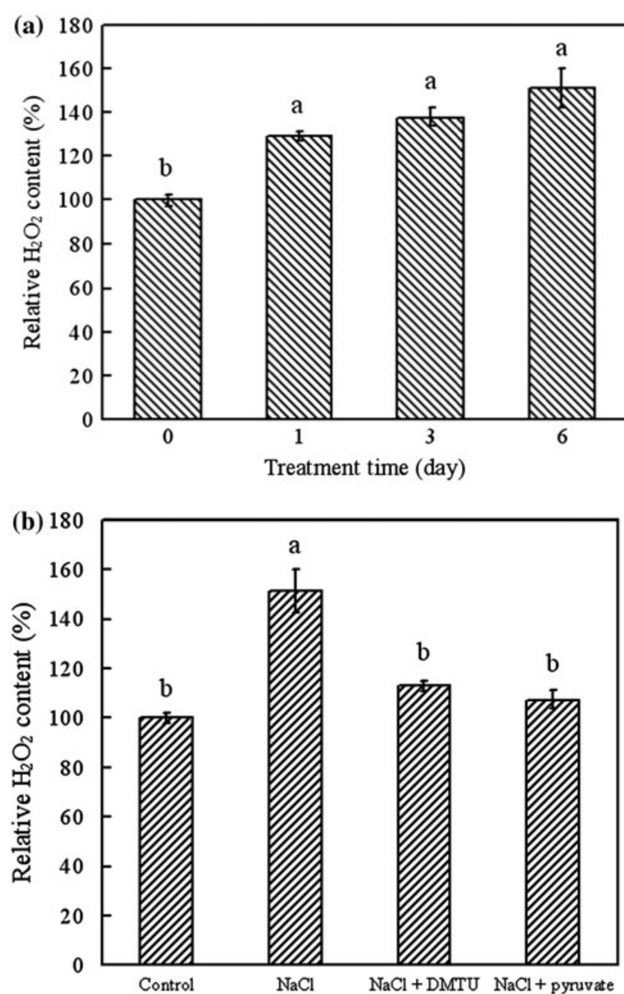

Fig. 2 **a** Levels of H_2O_2 in *Nitraria tangutorum* Bobr. calli under salinity stress. **b** Effects of dimethylthiourea (DMTU) or pyruvate on NaCl-induced increase of H_2O_2 content in *Nitraria tangutorum* Bobr. calli exposed to 75 mM NaCl for 6 days. Control content of H_2O_2 (100 %) corresponded to 94.86 ± 9.62 ng g^{-1} Fw and 56.61 ± 1.25 ng g^{-1} Fw in untreated calli growing for 3 and 6 days, respectively. Values represent the means of at least three replicates for each treatment, *bars* with *different letters* are significantly different at the 0.05 level

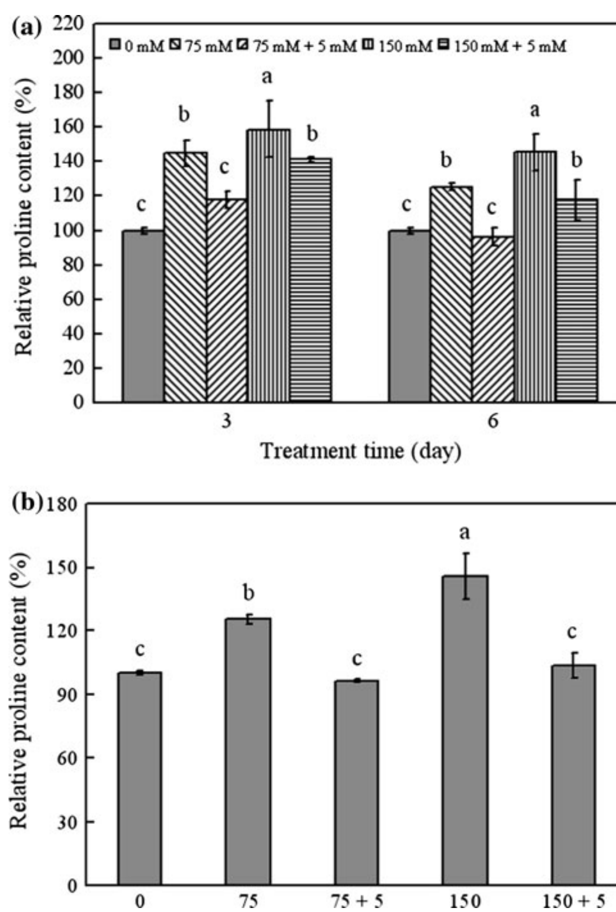

Fig. 3 Effects of 5 mM dimethylthiourea (DMTU, **a**) or 5 mM pyruvate (**b**) on salinity-induced elevation of proline level in calli of *Nitraria tangutorum* Bobr. after treatment for 3 or 6 days. 0, Control; 75, 75 mM NaCl; 150, 150 mM NaCl; 5, 5 mM pyruvate. Values represent the means of at least three replicates for each treatment, *bars* with *different letters* are significantly different at the 0.05 level

Discussion

Proline is an organic molecule and accumulates in plants when exposed to stress conditions including salinity (Zhao et al. 2009; Wang et al. 2011). In accordance with the research results, the present data exhibited notable increases in proline levels in *Nitraria tangutorum* Bobr. calli growing on medium containing different NaCl concentrations (Fig. 1a). Because proline can provide protection for plants against osmotic stress, oxidative damage and membrane integrity (Ozden et al. 2009; Pei et al. 2010), proline accumulation is believed to represent an important cellular mechanism for salinity tolerance (Kavi Kishor et al. 2005). More recently, a correlation between the proline accumulation and abiotic stress tolerance has been suggested in different halophytes and cell cultures (Lokhande et al. 2010; Sekmen et al. 2012). However, in *Hordeum vulgare* L., proline content is not correlated with salt tolerance (Patterson et al. 2009). Furthermore, Lv et al. (2011) reported that proline-overproducing plants showed the growth inhibition and the lower survival rate in response to stress environment. Therefore, further studies will be needed to better understand the relationship between proline accumulation and salinity tolerance in *Nitraria tangutorum* Bobr. calli.

The enhancement of proline content related to stress conditions has been suggested to achieve through different metabolic mechanisms, such as proline biosynthesis via both glutamate and ornithine pathways. Some investigations support the conclusion that the glutamate pathway, rather than the ornithine pathway, plays a vital role in proline accumulation (Roosens et al. 1999; Zhen and Ma 2009) because increased GK and decreased OAT activities

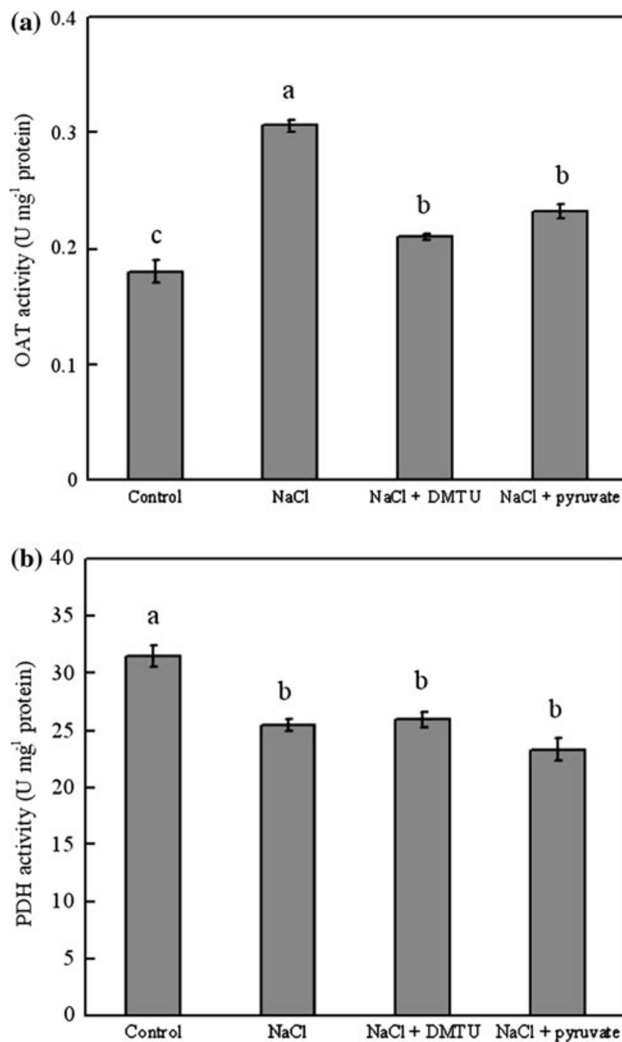

Fig. 4 Effects of dimethylthiourea (DMTU) or pyruvate on salinity induced changes of OAT (**a**) and PDH (**b**) activities in calli of *Nitraria tangutorum* Bobr.. Calli were treated with 75 mM NaCl for 6 days in the absence or presence 5 mM DMTU or 5 mM pyruvate, and the enzyme activities were assayed. Values represent the means of at least three replicates for each treatment, *bars* with *different letters* are significantly different at the 0.05 level

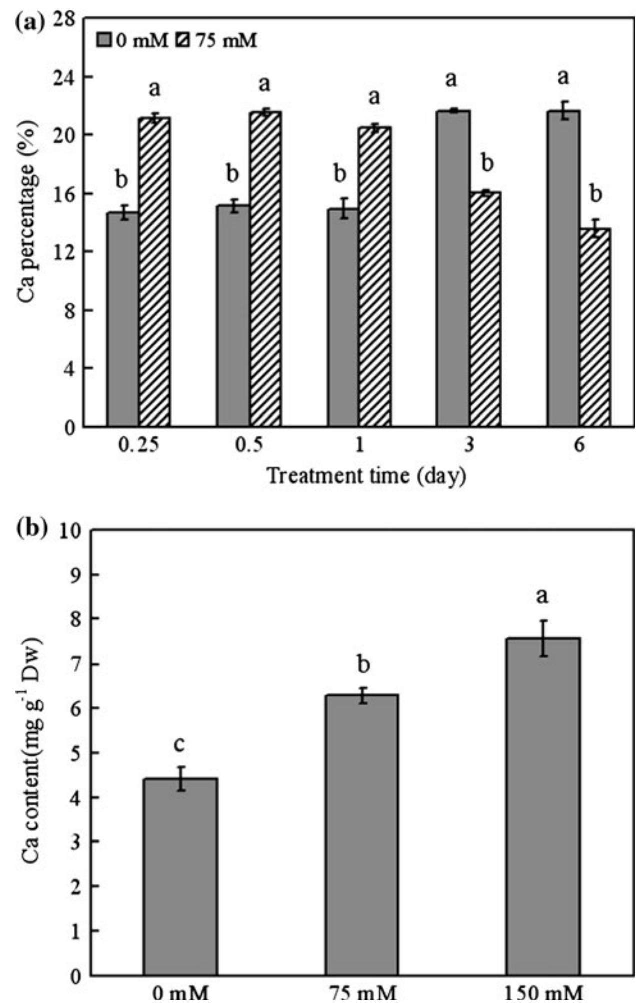

Fig. 5 **a** Determination of calcium (Ca) percentage in *Nitraria tangutorum* Bobr. calli with X-ray microanalysis. **b** Measurements of Ca^{2+} contents in *Nitraria tangutorum* Bobr. calli treated with NaCl for 1 day using an Inductive Coupled Plasma Emission Spectrometer. Values represent the means of at least three replicates for each treatment, *bars* with *different letters* are significantly different at the 0.05 level

were observed when plants were exposed to stress conditions. The study of Funck et al. (2008) showed that proline content is not affected in OAT *Arabidopsis* mutants, indicating that OAT is not related to proline accumulation. However, Krell et al. (2007) reported that the ornithine pathway, via an increase of OAT activity, contributes to proline synthesis in plants exposed to salinity treatment. In agreement with this finding, the present data showed that NaCl induced significant elevation of OAT activity but notable decrease of GK activity in *Nitraria tangutorum* Bobr. calli, suggesting that the ornithine pathway involving in the stimulation of OAT may be responsible for proline accumulation in response to NaCl treatment. Apart from proline biosynthesis, proline content is also determined by proline catabolism occurring in mitochondrial via the action of the enzyme PDH (Szabados and Savouré 2010). The studies of Kumar et al. (2003) and Wang et al. (2011) indicated a positive correlation between increased proline content and reduced PDH activity in response to salt stress in plant seedlings. In *Nitraria tangutorum* Bobr. calli, the treatment with NaCl for 3 or 6 days resulted in a significant decrease in PDH activity, but a significant increase in this enzyme activity was observed when calli were exposed to 75 mM NaCl for 9 days (Fig. 1d). Moreover, the changes of increase rate in OAT activity and decrease rate in PDH activity were correlated with the increase rate of proline content in calli at the same period of NaCl stress. Thus, the present data confirmed that in *Nitraria tangutorum* Bobr. calli the enhancement of proline synthese via the stimulation of OAT activity and the reduction of proline

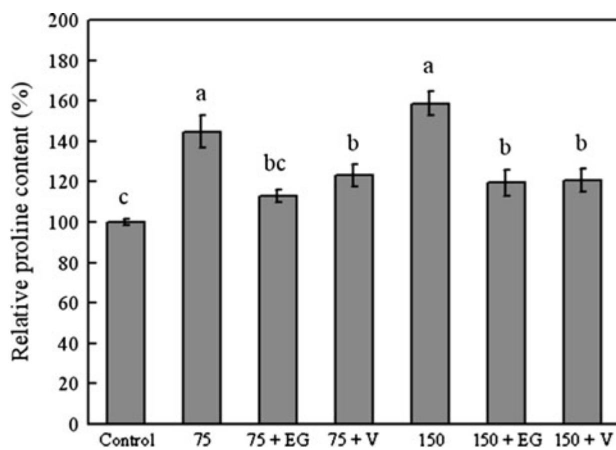

Fig. 6 Effects of EGTA and verapamil on salinity-induced enhancement of proline content in calli of *Nitraria tangutorum* Bobr. after treatment for 3 days. 75, 75 mM NaCl; 150, 150 mM NaCl; EG, 5 mM EGTA; V, 10 μM verapamil. Values represent the means of at least three replicates for each treatment, *bars* with *different letters* are significantly different at the 0.05 level

catabolism via the inhibition of PDH activity contribute to proline accumulation under salinity stress.

H_2O_2 accumulation is caused when plants are exposed to abiotic stresses including salinity (Hasegawa et al. 2000). *Nitraria tangutorum* Bobr. calli treated with 75 mM NaCl showed an increased H_2O_2 production with the duration of salt stress (Fig. 2a). Excessive H_2O_2 in cells can lead to oxidative stress, which damages biological membranes and some larger molecules (Forman 2007). On the other hand, H_2O_2 can be viewed as a signal molecule involved in different physiological processes (Forman 2007; Yang et al. 2007). A recent study has suggested that H_2O_2 may participate in signal transduction events involved in the regulation of proline metabolism (Yang et al. 2009). Differently, proline accumulation and decreased H_2O_2 generation in response to different stresses were observed by Simova-Stoilova et al. (2008) and Azzedine et al. (2011). By measuring the amount of proline in the absence or presence of 5 mM DMTU (a hydroxyl radical and H_2O_2 scavenger that is able to inactivate ROS) and pyruvate (an efficient and specifical H_2O_2 scavenger via oxidative dephosphorylation), our investigation indicated that proline accumulation was a consequence of increasing H_2O_2 generation from salinity-stressed calli. It was further supported by the decreased H_2O_2 level in calli treated with NaCl together DMTU or pyruvate in comparison with calli treated with salinity alone (Fig. 2b). Moreover, no significant effects on the activities of proline metabolic enzymes were observed in calli under DMTU or pyruvate treatments alone (data not shown). However, these two regents prevented the enhancement of OAT activity but did not affect the PDH activity induced by NaCl treatment for 6 days (Fig. 4). According to the preceding findings, we conclude that proline accumulation in response to the increase of H_2O_2 generation might be caused by the stimulation of OAT in salinity-treated calli.

Serving as an important messenger, Ca plays a fundamental role in regulating polar growth of cells and tissues as well as participates in plant adaptation to various stress factors including salinity stress (Lee and Liu 1999; Song et al. 2008). It has been indicated that Ca^{2+} supplement enhances NaCl stress-induced proline accumulation in *Oryza sativa* L. spp. indica (Cha-um et al. 2012) and in *Sorghum bicolor* roots (Colmer et al. 1996). However, the addition of 10 mM Ca^{2+} together with NaCl treatment reduces the accumulation of proline in leaf tissues of red-osier dogwood (Renault and Affifi 2009). These studies provide the evidence that exogenous Ca supplement can regulate proline levels in high plants exposed to salinity stress, whereas the conclusions are different, and even contradictory. Moreover, a previous study has indicated that endogenous Ca^{2+} is negatively associated with NaCl-induced proline accumulation in *U. fasciata* (Lee and Liu 1999). In the present investigation, Ca^{2+} chelator EGTA could reverse the enhanced proline content after treatment with 75 mM NaCl for 3 days (Fig. 6), suggesting that Ca might be related to proline accumulation in *Nitraria tangutorum* Bobr. calli when exposed to salinity. High salinity can regulate the concentration of Ca^{2+} in plant cells and tissues (Halperin et al. 1997). Increased Ca content in plants during salt stress was reported by different authors (Gao et al. 2004; Yang et al. 2007). In contrast to these conclusions, Lee and Liu (1999) observed that increasing NaCl concentration could result in a fast decrease in the amount of Ca^{2+} in *U. Fasciata*. In *Nitraria tangutorum* Bobr. calli, we found a significantly enhanced Ca content during the early stage of 75 mM NaCl treatment (from 6 h to 24 h), but with the duration of salinity treatment (3 and 6 days) increasing, there was a decrease of Ca content in calli (Fig. 5a). It has been indicated that increased cytosolic Ca^{2+} may be induced by Ca^{2+} influx to the cytosol from the apoplast across the plasma membrane (White and Broadley 2003). In this study, 10 μM verapamil, a Ca channel blocker, also reduced the increase of proline level in calli treated by salinity (Fig. 6). This result further demonstrated that Ca signal via the influx of Ca^{2+} from the apoplast across the plasma membrane was responsible for proline accumulation in response to salinity. Based on the above results, we presumed that the enhancement in Ca content might be attributed to the increased generation of H_2O_2 from salinity-stressed calli, and that proline accumulation was partly due to the increased Ca content in response to NaCl treatment.

In conclusion, our results indicate that NaCl treatment induces increased level of proline in *Nitraria tangutorum* Bobr. calli, and this is due to the enhancement of OAT activity and the decrease of PDH activity in response to salinity. In addition, the increased Ca signal during the early stage of NaCl treatment and the stimulation of OAT resulting from the elevation of H_2O_2 generation are essential for proline accumulation in salinity-stressed calli.

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