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Research Article

Exogenous proline mitigates the detrimental effects of saline and alkaline stresses in *Leymus chinensis* (Trin.)

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Abstract Proline accumulates in plants under environmental stresses including saline stress and alkaline stress. Here, we investigated the responses to two different stresses, saline stress (200 mM NaCl) and alkaline stress (100 mM Na_2CO_3) in two *Leymus chinensis* (Trin.) genotypes, LcWT07 and LcJS0107, and effects of exogenous proline on the activities of antioxidant enzymes. Both saline stress and alkaline stress significantly induced the accumulation of proline in leaves of the two genotypes after 96 h, and alkaline stress caused a transient and significant increase in LcJS0107 plants at 6 h. A reduction in the activities of catalase (CAT, EC 1.11.1.6) and ascorbate peroxidase (APX, EC 1.11.1.11), but not in the activity of superoxide dismutase (SOD, EC 1.15.1.1), was detected in plants exposed to saline and alkaline stresses. Remarkable decrease in relative water contents (RWC) was found in 144 h stressed plants. However, lipid peroxidation estimated by malonyldialdehyde (MDA) content in leaves remained relatively stable. With the addition of exogenous proline, it did not cause changes of proline levels in two genotypes, but combined with saline or alkaline stress, the exogenous application of proline significantly induced proline accumulation after even short treatment periods. Combined with salt stress, the exogenous application also increased the activities of CAT and APX. These results indicated that exogenous proline not only increases proline levels in vivo as a osmotic adjustment under stress, but mitigates the detrimental effects of saline and alkaline stresses by increasing the activities of antioxidant enzymes.

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Introduction

Osmotic stress caused by salinity severely limits crop productivity (Boyer 1982). To counteract osmotic stress, many plants accumulate several kinds of compatible solutes such as proline, glycinebetaine, sugars and polyols (Flowers et al. 1977). Proline as a well-known compatible solute, plays a pivotal role in the process of osmotic adjustment in plants by helping to maintain sufficient cell turgor for growth to proceed (Zimmermann 1978). It also is reported to protect enzymes against abiotic stresses (Pollard and Wyn Jones 1979) and stabilize many functional units such as complex II electron transport, membranes and proteins, and enzymes such as RuBisco to enhance stress tolerance (Mäkelä et al. 2000; Hamilton and Heckathorn 2001). In addition, the accumulation of proline generally occurring in many plant species under stress conditions has been correlated with tolerance to stresses, such as salinity, drought, freezing, and oxidative stress (Delauney and Verma 1993; Chen et al. 2006). However, Lutts et al. (1999) have reported that accumulation of proline under salt stress in leaves of rice is a symptom of salt injury rather than an indication of salt tolerance. Therefore, further studies are needed to determine whether the relationship between stress tolerance and the accumulation of proline is species-specific or if it can be altered by experimental conditions.

Exogenous proline has been found to play roles in the form of either osmoprotection or cryoprotection (Songstad et al. 1990). Roy et al. (1979) have reported that exogenous application of 30 mM proline counteracted the adverse effects of salinity on early seedling growth. Heyser et al. (1989) have reported that exogenous application of 5 mM

proline inhibited the metabolism of ^{13}C-glutamic acid to proline in a cell suspension of halophilic *D. spicata* exposed to NaCl. Moreover, Okuma et al. (2004) have suggested that exogenous proline reduces the oxidation of lipid membranes under stress conditions and protects enzymes and cell membranes. Hoque et al. (2007) have suggested that exogenous proline increases the activities of most enzymes involved in NaCl-induced ASC-GSH cycle, and improves salt tolerance in tobacco. However, Hellmann et al. (2000) have reported that even moderate concentrations of exogenous proline are toxic for *Arabidopsis* in axenic culture. Thus, the actual roles of exogenous proline taking in the stress defense process of plants still require further understanding.

The halophyte *Leymus chinensis* (Trin.) Tzvel., a perennial rhizome grass placed in tribe Gramineae, is a plant species that has adapted to thrive under natural stress conditions; it is widely distributed throughout northern China, Mongolia and Siberia (Huang et al. 2004). Because of its intrinsic tolerance of highly alkaline-sodic soil conditions (Jin et al. 2006), *L. chinensis* is used as a soil-binding plant to protect soil from desertification. Due to its high vegetative productivity and protein content, this species is used as a major forage product to meet the needs of grazing (Shu et al. 2005). However, climate changes, overgrazing and reclamation of grassland result in severe deterioration of the grassland ecosystem, which significantly affects human life and causes other ecological problems. Thus, a greater understanding of stress tolerance mechanisms would be helpful for improving the traits of this grass.

The present study was, therefore, conducted to evaluate the responses to saline and alkaline stresses on proline accumulation, and the activities of antioxidant enzymes in two halophyte *L. chinensis* genotypes and the effects of exogenous proline on these responses.

Materials and Methods

Plant material and growth conditions

Mature seeds of LcWT07 *L. chinensis* plants were obtained from natural grassland in Siping, Jilin, China; these seeds have been optimized for environment by many years of natural evolution. Mature seeds of LcJS0107 *L. chinensis* plants that were cultivated as a new variety having high tolerance and vegetative productivity, were collected from the Jisheng Chinese Wildrye Excellent Seed Station, Changchun, Jilin, China. Mature seeds of both genotypes were dipped with water in 4°C for 3 days, and those sinking to the bottom were selected and grown in pots containing clay/vermiculite (3/1, v/v). The cultures were grown one plant per pot in a 25°C greenhouse with a 16/8 h (day/night) photoperiod and a relative humidity between 45% and 70%. Seedlings germinated in these conditions were watered daily with Hoagland nutrient solution (Hoagland and Arnon 1950).

Saline and alkaline stress treatments

Six-week-old LcWT07 and LcJS0107 plants cultured in the above conditions were subjected to saline (200 mM NaCl) or alkaline stress (100 mM Na_2CO_3) with different treatment times (0, 1, 3, 6, 12, 24, 48, 96, 120, 144 h) and sampled. To evaluate the toxicity of exogenous proline, 6-week-old plants were watered with various concentrations of proline solutions (0, 50, 100, 200, 500 mgl^{-1}) without any additional salt stress for 6 h and sampled. To test the effect of exogenous proline, 6-week-old plants were watered with certain concentration of proline solution combined with saline or alkaline stress for 6 h and 72 h and sampled, respectively. Three independent repeats were performed for each experiment. Ten plants were analyzed in for each experiment.

Plant growth

The aerial and underground parts of 6-week-old LcWT07 and LcJS0107 *L. chinensis* plants treated with saline and alkaline stresses were sampled at various treatment times (0, 24, 72, 144 h) to determine the fresh weight (FW). For dry weight (DW) determination, the aerial and underground parts were dried in hot air oven at 60°C for 72 h and weighed. The relative water contents (RWC) were calculated using the formula:

$$RWC = (FW - DW) \times 100\% / FW$$

Physiological characteristics of the leaves of LcWT07 and LcJS0107 exposed to saline and alkaline stresses were determined at the point of death caused by stress. Three independent experiments were performed for each condition, and at least ten plants were analyzed for this experiment.

Estimation of proline

Proline content in the leaves was measured by using modified colorimetric methods (Bates et al. 1973). Fresh

leaf tissue (0.5 g) was triturated in liquid nitrogen, and homogenized with methanol/chloroform/water extraction solution (12/5/1, v/v/v). Then extraction solution was centrifuged at 5,000 rpm for 5 min. A solution containing 2 ml of glacial acetic acid and 2 ml acid ninhydrin solution were added to 2 ml of centrifuged extraction solution and incubated for 1 h in a boiling water bath followed by cooling in ice bath. A 3 ml aliquot of toluene was then added, and the sample was mixed vigorously. The chromophore containing toluene was aspirated from aqueous phase and the absorbance was measured at 518 nm (NanoPhotometer™ IMPLEN). The amount of proline was calculated using a standard graph of known concentrations.

Antioxidant activities

Fresh leaf tissues were ground in a mortar with liquid nitrogen and then homogenized in the respective extraction buffer. Homogenates were centrifuged at 12,000 rpm at 4°C for 20 min. The extract from the extraction solution was used for enzyme activity assay.

Superoxide dismutase (SOD, EC 1.15.1.1) Fresh leaf tissues (0.2 g) were homogenized in a mortar with 2 ml of extraction medium containing 50 mM potassium phosphate buffer (pH 7.0) and 0.1 mM EDTA. SOD activity was determined according to the method of Beauchamp and Fridovich (1971) following the photo-reduction of nitroblue tetrazolium (NBT). The reaction mixture contained: 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 μM NBT, 2 μM riboflavin and 100 μl of enzyme extract. Riboflavin was added as the last component and the reaction was initiated by placing the tubes under 15 W fluorescent lamps. The reaction was terminated after 10 min by removing the reaction tubes from the light source. Non-illuminated and illuminated reactions without enzyme extract served as calibration standards. SOD activity was measured by the absorbance at 560 nm. One unit of SOD was defined as the enzyme activity which inhibited the photoreduction of NBT to blue formazan by 50%, and SOD activity of the extracts was expressed as SOD units mg protein^{-1} min^{-1}.

Catalase (CAT, EC 1.11.1.6) Fresh leaf tissues (0.2 g) were homogenized in a mortar with 2 ml of extraction medium containing 50 mM potassium phosphate buffer (pH 7.0) and 0.1 mM EDTA. The reaction medium contained 50 mM potassium phosphate buffer (pH 7.0), 12.5 mM H_2O_2, and 50 μl of enzyme extract (Beers and Sizer 1952). CAT activity was determined by following the decrease in absorbance at 240 nm, and was expressed as nmole of H_2O_2 decomposed mg protein^{-1} min^{-1}.

Ascorbate peroxidase (APX, EC 1.11.1.11) Fresh leaf tissues (0.2 g) were homogenized with 0.8 ml of extraction medium containing 50 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.1 mM H_2O_2 (Nakano and Asada 1981). The reaction medium contained 50 mM Tris-HCl (pH 7.8), 0.5 mM ascorbate, 0.1 mM EDTA, 0.1 mM H_2O_2 and 50 μl of enzyme extract for a total volume of 1 ml. The activity was followed by the decrease in absorbance at 290 nm, and was expressed as μmole of ascorbate oxidized mg protein^{-1} min^{-1}. APX activity was determined using an extinction coefficient of 2.8 mM^{-1} cm^{-1}.

Lipid peroxidation

Lipid peroxidation was estimated by determining the malonyldialdehyde (MDA) content of the leaves (Zobayed and Saxena 2003). Fresh leaf tissues (0.5 g) were homogenized in 2 ml of 0.1% trichloroacetic acid (TCA). A 0.5 ml sample of enzyme extract was mixed with 1.5 ml of 0.5% thiobarbituric acid (TBA) prepared in TCA 20%, and incubated at 90°C for 20 min. After stopping the reaction in an ice bath, samples were centrifuged at 8,000 rpm for 5 min., and the supernatant absorbance at 532 nm was then measured. After subtracting the non-specific absorbance at 600 nm, MDA concentration was determined using the extinction coefficient 155 mM^{-1} cm^{-1}.

Statistical analysis

Statistically significant differences between means were determined by two-way analysis of variance (ANOVA) using Duncan's multiple-range test (Duncan 1955). A P value of less than 0.05 was considered significant.

Results

Saline and alkaline stress effects on proline accumulation

Proline accumulation was observed in leaves of two 6-week-old *L. chinensis* genotypes treated with 200 mM NaCl and 100 mM Na_2CO_3 for various times (Fig. 1). High levels of proline did not accumulate in LcWT07 and LcJS0107 until 96 h after stress. At 96 h after NaCl stress proline levels in LcWT07 increased significantly ($P < 0.05$) compared to those in normal conditions and reached the maximum of proline accumulation at 120 h after stress, with an 18.40-fold increase. During the

following 24 h of stress, the levels of proline accumulation showed a significant decrease compared to the maximum at 120 h after stress (Fig. 1A). Proline accumulation in leaves of LcJS0107 significantly increased ($P < 0.05$) and reached the maximum at 120 h after stress, with a 24.54-fold increase compared to the control. During the following 24 h of stress, proline accumulation in the leaves remained at a relatively high level in LcJS0107, but not in LcWT07 (Fig. 1A).

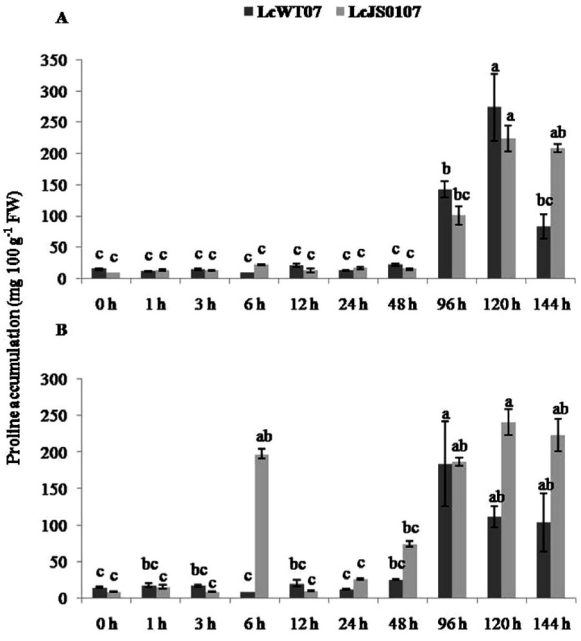

Fig. 1 Proline accumulation in leaves of LcWT07 and LcJS0107 plants under saline stress (A) and alkaline stress (B) as a function of time. Values are the mean of three repeated experiments. Means followed by the same letter in the same genotype are not significantly different at $P < 0.05$ according to two-way ANOVA using Duncan's multiple-range test

Na_2CO_3 stress significantly induced proline accumulation in leaves of LcWT07 and LcJS0107 after 96 h of stress ($P < 0.05$, Fig. 1B). The maximum levels of proline accumulation of 12.39- and 26.39-fold were reached 96 h after stress for LcWT07 and 120 h after stress for LcJS0107, respectively. To be mentioned, LcJS0107 showed a transient and significant increase in proline accumulation at 6 h after stress, and then decreased rapidly to normal levels (Fig. 1B). At the late stage of stress, LcJS0107 still remained high levels of proline, and downtrend of proline accumulation began to appear in LcWT07.

Saline and alkaline stress effects on growth status, relative water contents and lipid peroxidation

The new variety LcJS0107 used in this study showed stronger stress adaptation than Chinese wild-type LcWT07, with one more day longevity (data not shown). Exposed to 200 mM NaCl and 100 mM Na_2CO_3 stresses, this grass showed different morphological responses at the point of death, with no variation between both genotypes. Leaves kept viridian at the base under NaCl stress, and turned yellow from the tip towards to the base and seared to die (Fig. 2), while leaves under Na_2CO_3 stress displayed deep green at the base and also turned yellow at the tip, and then died (Fig. 2).

The relative water contents (RWC) of the aerial and underground parts of LcWT07 and LcJS0107 exposed to saline and alkaline stresses decreased with longer treatment times (Fig. 3). NaCl stress did not affect RWC of the aerial and underground parts of LcWT07 and LcJS0107 until 72 h of stress. After 144 h after stress, both genotypes showed a significant decrease in RWC

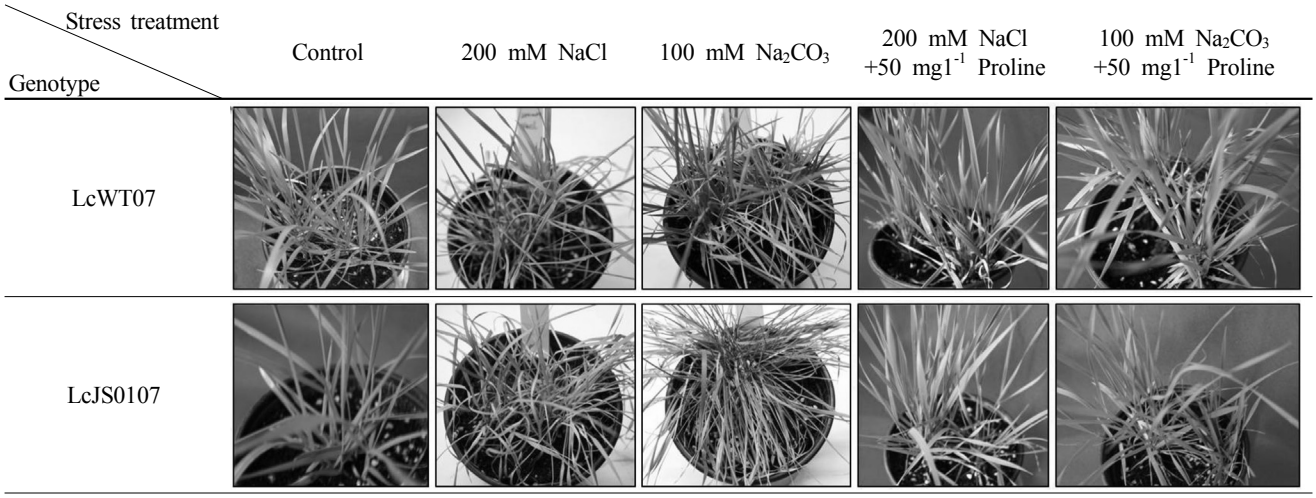

Fig. 2 The morphological characteristics of the halophytic *L. chinensis* exposed to 200 mM NaCl and 100 mM Na_2CO_3 stresses at the point of death, and exogenous application of 50 mgl^{-1} proline with 200 mM NaCl and 100 mM Na_2CO_3

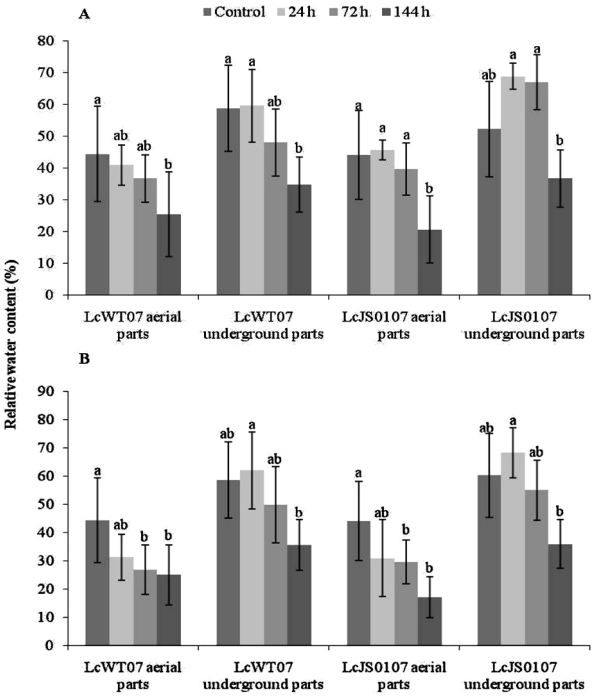

Fig. 3 Relative water contents (RWC, %) of the aerial and underground parts of LcWT07 and LcJS0107 under saline stress (A) and alkaline stress (B) at various treatment times (0, 24, 72, 144 h). Values are the mean of three repeated experiments. Means followed by the same letter in the same group are not significantly different at $P < 0.05$ according to two-way ANOVA using Duncan's multiple-range test

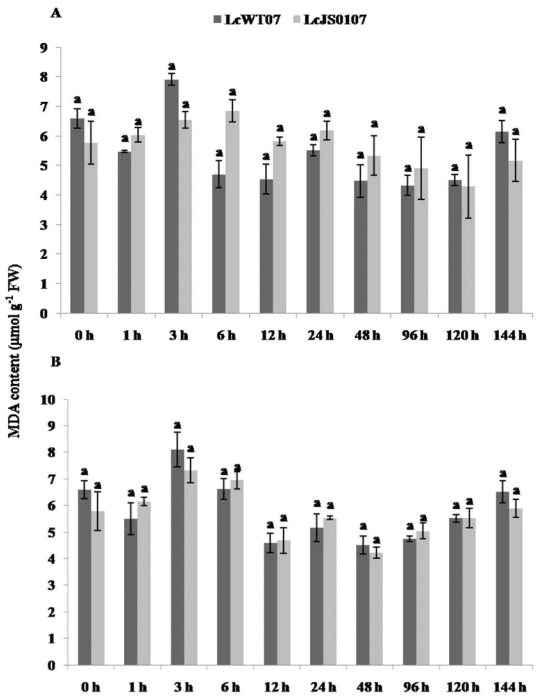

Fig. 4 Lipid peroxidation evaluated by MDA content in leaves of LcWT07 and LcJS0107 plants under saline stress (A) and alkaline stress (B) as a function of time. Values are the mean of three repeated experiments. Means followed by the same letter in the same genotype are not significantly different at $P < 0.05$ according to two-way ANOVA using Duncan's multiple-range test

($P < 0.05$, Fig. 3A). Under Na_2CO_3 stress, RWC of underground parts of both genotypes was not found to have significant differences over 72 h of stress, but the RWC of the aerial parts had a significant decrease after 72 h compared to the control levels ($P < 0.05$, Fig. 3B). Aerial parts of both genotypes had continuous decline of RWC at 144 h after alkaline stress, while underground parts also appeared remarkable decrease in RWC after 144 h of stress.

Lipid peroxidation evaluated by MDA content in both genotypes was investigated under saline stress (Fig. 4A) and alkaline stress (Fig. 4B). Except a slight increase in MDA levels appeared after 3 h of stress, over the 144 h of experimental period, relatively invariable MDA contents were observed in both genotypes.

Saline and alkaline stress effects on antioxidant activities

NaCl stress caused a significant increase in CAT activity ($P < 0.05$) at 3 h after stress in LcWT07 and LcJS0107, respectively, with 11.44- and 10.02-fold higher activity compared to control levels (Fig. 5A). LcJS0107 maintained relatively higher CAT activity until 6 h after stress, while LcWT07 showed a large decrease. As the treatment time increasing, CAT activities in both genotypes returned to normal levels comparable to the control and showed a downtrend at 144 h of stress. Under Na_2CO_3 stress, CAT activity in LcWT07 began to increase after 12 h of stress, while LcJS0107 showed an earlier significant increase in CAT activity at 6 h than LcWT07 and maintained relatively high CAT activity until 12 h after stress (Fig. 5B). At the late stage of stress, both genotypes also showed decreased CAT activity.

In this work, very low APX activities were observed in LcWT07 and LcJS0107 (Fig. 5C, D). Under NaCl stress, APX activities in LcWT07 and LcJS0107 were slightly increased after 1 h of stress and then decreased with complicated fluctuations over the course of the experiment. After 24 h of NaCl stress, APX activities in both genotypes were highly decreased and remained relatively low levels until 144 h (Fig. 5C). Under Na_2CO_3 stress, there was also a fluctuant status in APX activity at the early stage of stress, with a remarkable decrease at 1 h after stress, but a significant increase ($P < 0.05$) at 24 h after stress (Fig. 5D). Though APX activities in both genotypes under alkaline stress changed randomly, the overall trend of the activity

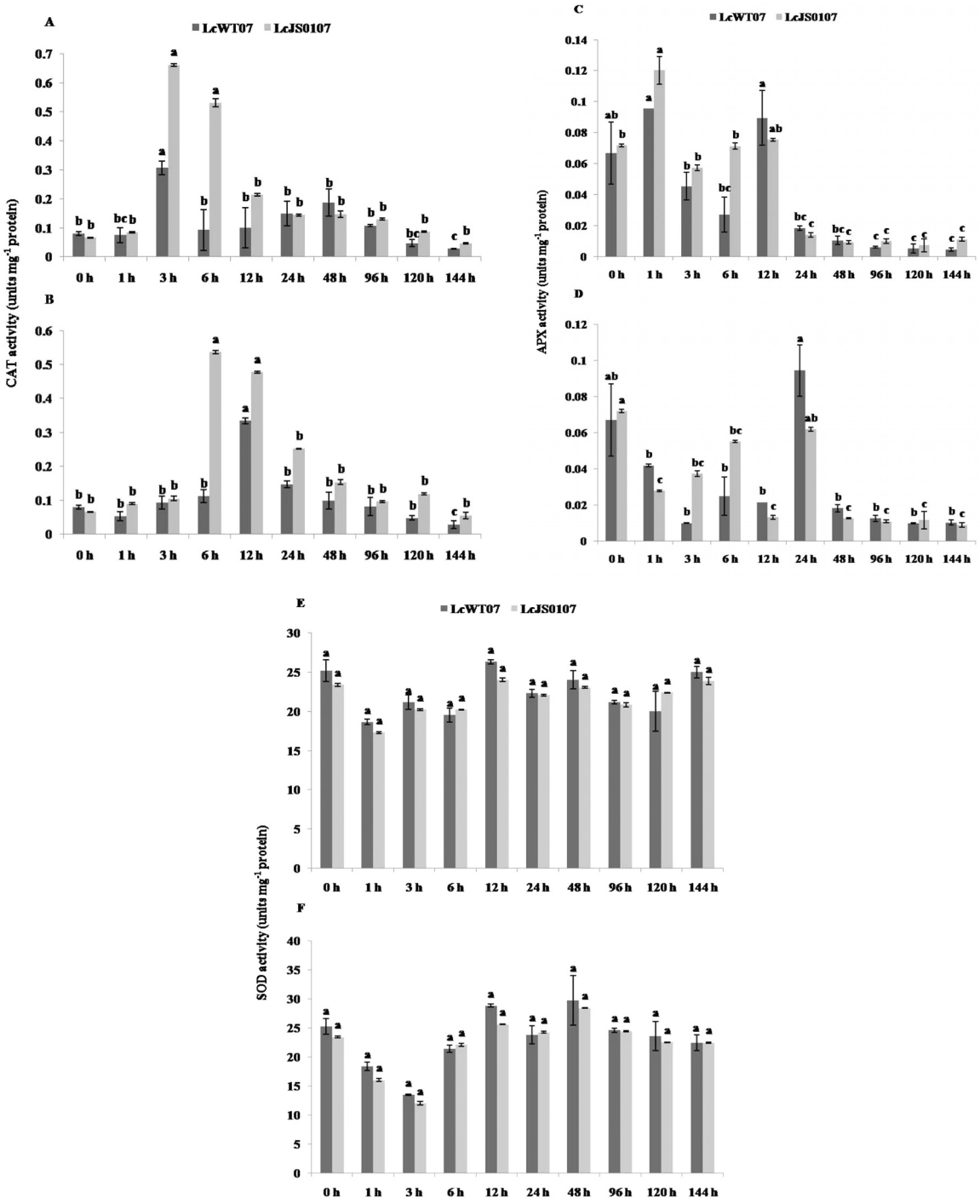

Fig. 5 Antioxidant activities of CAT (A and B), APX (C and D), and SOD (E and F) in leaves of LcWT07 and LcJS0107 plants under stresses [saline stress (A, C, E) and alkaline stress (B, D, F), respectively]. Values are the mean of three repeated experiments. Means followed by the same letter in the same genotype are not significantly different at $P < 0.05$ according to two-way ANOVA using Duncan's multiple-range test

was decreasing after 48 h with increasing treatment time. Either NaCl or Na_2CO_3 stress did not cause remarkable changes in SOD activity of both genotypes during 144 h of stress (Fig. 5E, F). However, there was a slight decrease in SOD activity over the first 3 h of Na_2CO_3 stress, with 53.31% and 51.27% of the activity before stress in LcWT07 and LcJS0107, respectively.

Protective effects of exogenous proline

To determine optimal concentrations of exogenous proline not to cause additional and potential stress damage, proline

accumulation was evaluated in LcWT07 and LcJ0107 watered exogenously with various concentrations of proline solutions (0, 50, 100, 200, 500 mgl^{-1}, Fig. 6). All exogenous proline did not induce increase in proline accumulation compared to the control level. Based on non-additional effects of exogenous proline on proline accumulation in vivo, 50 mgl^{-1} exogenous proline was selected for further experiments. With the addition of 50 mgl^{-1} exogenous proline combined with saline or alkaline stress, it did not enhance significantly proline accumulation, irrespective in stress type and genotype type ($P < 0.05$, Fig. 7), but also highly improve growth status (Fig. 2). Although alkaline stress caused a transient increase in LcJS0107 after 6 h of stress (Fig. 1B, 7), with exogenous application of proline, it aggravated the increased proline levels significantly compared to levels under alkaline stress ($P < 0.05$, Fig.7). And the increased proline levels induced by alkaline stress in both genotypes were higher than those induced by saline stress.

To investigate whether exogenous proline combined with stress also affected lipid peroxidation and antioxidant activities, MDA contents, CAT, APX, and SOD activities of both genotypes were assayed (Fig. 8). MDA contents in both genotypes did not show significant differences with the addition of exogenous proline under either saline or alkaline stress (Fig. 8A). However, a significant increase in CAT activity was observed in LcWT07 after 6 h saline stress or

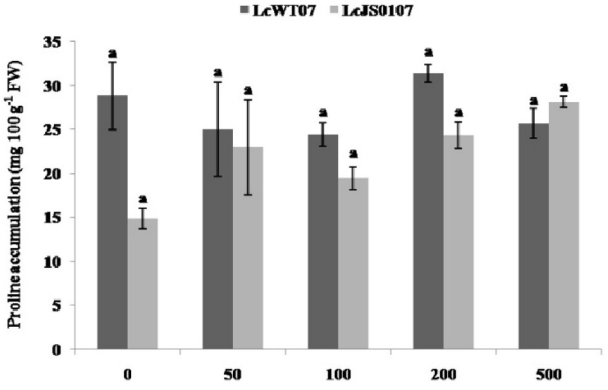

Fig. 6 Proline accumulation in leaves of LcWT07 and LcJS0107 plants with the addition of various concentrations of exogenous proline (0, 50, 100, 200, 500 mgl^{-1}) for 6 h. Values are the mean of three repeated experiments. Means followed by the same letter in the same genotype are not significantly different at $P < 0.05$ according to two-way ANOVA using Duncan's multiple-range test

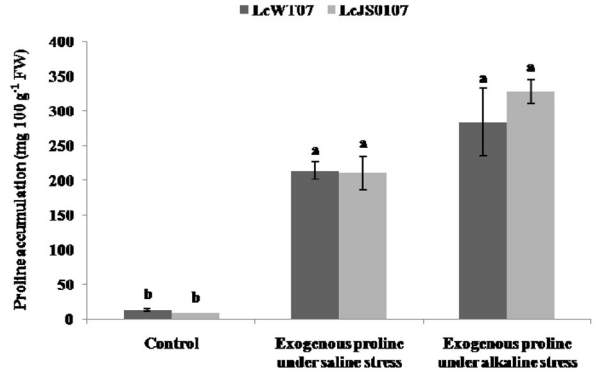

Fig. 7 Proline accumulation in leaves of LcWT07 and LcJS0107 plants with the addition of 50 mgl^{-1} exogenous proline combined with saline or alkaline stress for 6 h. Control represented here is the value from non-stress conditions. Values are the mean of three repeated experiments. Means followed by the same letter in the same genotype are not significantly different at $P < 0.05$ according to two-way ANOVA using Duncan's multiple-range test

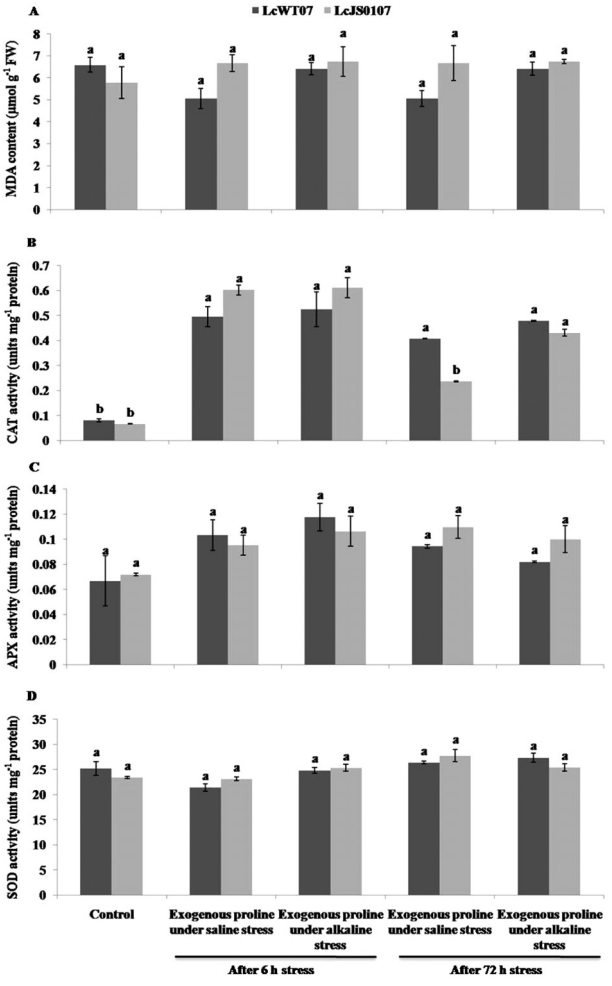

Fig. 8 Lipid peroxidation evaluated by MDA content (A), and antioxidant activities of CAT (B), APX (C) and SOD (D) in leaves of LcWT07 and LcJS0107 plants with the addition of 50 mgl^{-1} exogenous proline combined with saline or alkaline stress for 6 h and 72 h. Control represented here is the value from non-stress conditions. Values are the mean of three repeated experiments. Means followed by the same letter in the same genotype are not significantly different at $P < 0.05$ according to two-way ANOVA using Duncan's multiple-range test

alkaline stress in the presence of exogenous proline, but not in LcJS0107 (Fig. 1, 8B). When exposed to 72 h stress with the exogenous application of proline, LcWT07 continued to maintain high levels of CAT activity, while LcJS0107 only showed a remarkable increase in CAT activity under 72 h alkaline stress. Only slight increases were observed in APX activity of both genotypes when stressed with the addition of exogenous proline for 6 h and even 72 h (Fig. 8C). Moreover, the exogenous proline combined with stress did not cause any changes in SOD activity over the 72 h experimental period (Fig. 8D).

Discussion

Proline accumulation occurs in some plants under environmental stress conditions, which is reported to be involved in enhanced stress tolerance and protection from stress damage (Rhodes and Hanson 1993). Proline dose not act as only a nitrogen source during the growth of plant cells, but plays a protective role against salt stress (Okuma et al. 2000). However, it is also reported that the increased levels of proline are not enough to adjust the osmotic potential in some plants under stress conditions (Hamilton and Heckathorn 2001), therefore exogenously adding proline has been used as a common way to supply more proline sources and successively enhance proline accumulation.

Various stresses result in the production of ROS including hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$), and the hydroxyl radical ($^{\bullet}OH$) in plants, which can cause oxidative damage to many cellular components including membrane lipids, proteins, and nucleic acids. Several enzymes are involved in the detoxification of ROS such as CAT, SOD, APXs, and POXs. Additionally, halophytic plants with the intrinsic adaptation have been considered as models for understanding stress defense mechanisms. In this study, we chose the halophyte *L. chinensis* as a model plant, and tested the stress-induced proline accumulation and effects of exogenous proline. Although the halophyte *L. chinensis* has been reported to be adapted under natural alkali-conditions of high pH value (Jin et al. 2006; Anamthawat Jónsson et al. 2009), the intrinsic stress adaptation depended on origin conditions and plant species. Chinese wild-type LcWT07 used in this study begins to appear kraurotic and effete after 144 h saline stress and alkaline stress in this work, while the new variety *L. chinensis* LcJS0107 appears the decay of vital force under both stresses one day later than LcWT07 (data not shown). And the intrinsic stress adaptation and stress tolerance of LcWT07 and LcJS0107 is being done by authors.

In this study, we observed a significant increase in proline accumulation in leaves of LcWT07 and LcJS0107 *L. chinensis* plants under 200 mM NaCl and 100 mM Na_2CO_3 stresses. Moreover, the reduction in RWC, and the decrease in antioxidant enzymes activities of CAT and APX were obtained during the experimental periods of stress treatment. However, our results showed that both stresses used in this study did not cause oxidative stress damage, with relatively stable levels of MDA contents in leaves of both genotypes, but only some stress defense responses. Interestingly, all changes in RWC and antioxidant enzymes activities were earlier and stronger in LcJS0107 than in LcWT07. Integrated with the early response of the transient and significant proline accumulation in LcJS0107 plants after 6 h of alkaline stress, LcJS0107 plant was believed to achieve higher stress tolerance by more sensitivity to stress, especially alkaline stress.

To investigate the correlation between exogenous proline and stress tolerance, we selected 6 h, a short period of stress treatment for evaluating the effects of exogenous proline. With the exogenous application of various concentrations of proline, changes of proline levels in vivo were not observed in leaves of both genotypes, however, combined with saline or alkaline stress, exogenous application of 50 mgl^{-1} proline induced significant increase in proline accumulation in both genotypes even after 6 h. Similar results had reported that exogenous proline improves the salt tolerance by increasing the intracellular accumulation of proline (Okuma et al. 2004; Hoque et al. 2007). Other responses to stress, such as reduced antioxidant activities were also highly improved by exogenous proline. Due to the sensitivity to stress, CAT activity in leaves of LcJS0107 showed high levels at 3 h and remained relatively high levels until 6 h after stress. With the addition of exogenous proline for 6 h, it did not induce the additional increase in CAT activity, just with primary high levels. However, the addition of exogenous proline for 72 h did cause a significant increase in CAT activity under alkaline stress. Moreover, LcWT07 plants had a significantly increase in CAT activity in the presence of exogenous proline, compared to the levels in the absence of exogenous proline during the 72 h experimental period. A slight decrease or invariability in APX activity was obtained in LcWT07 and LcJS0107 after 6 h of stress, whereas with the exogenous application of proline, APX activities were slightly enhanced. No difference in SOD activity was observed in both genotypes during these processes.

In conclusion, different responses to 200 mM NaCl and 100 mM Na_2CO_3 stresses and effects of exogenous proline in LcWT07 and LcJS0107 were investigated in this work.

A positive relationship between exogenous proline and antioxidant enzyme activities in *L. chinensis* has been observed through an increase in proline accumulation and enhancement of antioxidant enzyme activities. Therefore, we conclude that exogenous proline mitigates the detrimental effects of saline and alkaline stresses.

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References

Anamthawat Jónsson K, Wenke T, Thósrsson ÆTh, Sveinsson S, Zakrzewski F, Schimidt T (2009) Evolutionary diversification of satellite DNA sequences from *Leymus* (Poaceae: Triticeae). Genome 52:381-390

Bates LS, Waldern RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39:205-207

Beauchamp C, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44:276-287

Beers RF, Sizer IW (1952) A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J Biol Chem 195:133-140

Boyer JS (1982) Plant productivity and environment. Sci 218:443-448

Chen CB, Wanduragala S, Becker DF, Dickman MB (2006) Tomato QM-Like protein protects *Saccharomyces cerevisiae* cells against oxidative stress by regulating intracellular proline levels. Appl Environ Microbiol 72:4001-4006

Delauney AJ, Verma DPS (1993) Proline biosynthesis and osmoregulation in plants. Plant J 4:215-223

Duncan DB (1955) Multiple range and multiple *F* tests. Biometrics 11:1-42

Flowers TJ, Troke PF, Yeo AR (1977) The mechanism of salt tolerance in halophytes. Annu Rev Plant Physiol 28:89-121

Hamilton EW, Heckathorn SA (2001) Mitochondrial adaptations to NaCl. Complex I is protected by anti-oxidants and small heat shock proteins, whereas complex II is protected by proline and betaine. Plant Physiol 126: 1266-1274

Hellmann H, Funck D, Rentsch D, Frommer WB (2000) Hypersensitivity of an *Arabidopsis* sugar signaling mutant toward exogenous proline application. Plant Physiol 122:357-368

Heyser JW, De Bruin D, Kincaid ML, Johnson RY, Rodriguez MM, Robinson NJ (1989) Inhibition of NaCl-induced proline biosynthesis by exogenous proline in halophilic *Distichlis spicata* suspension cultures. J Exp Bot 40:225-232

Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. Calif Agric Exp Stn Circ 347:1-32

Hoque MA, Banu MNA, Okuma E, Amako K, Nakamura Y, Shimoishi Y, Murata Y (2007) Exogenous proline and glycinebetaine increase NaCl-induced ascorbate-glutathione cycle enzyme activities, and proline improves salt tolerance more than glycinebetaine in tobacco Bright Yellow-2 suspension-cultured cells. J Plant Physiol 164:1457-1468

Huang Z, Zhu J, Mu X, Lin J (2004) Pollen dispersion, pollen viability and pistil receptivity in *Leymus chinensis*. Ann Bot 93:295-301

Jin H, Plaha P, Park JY, Hong CP, Lee IS, Yang ZH, Jiang GB, Kwak SS, Liu SK, Lee JS, Kim YA, Lim YP (2006) Comparative EST profiles of leaf and root of *Leymus chinensis*, a xerophilous grass adapted to high pH sodic soil. Plant Sci 170:1081-1086

Lutts S, Majerus V, Kinet JM (1999) NaCl effects on proline metabolism in rice (*Oryza sativa*) seedlings. Physiol Plant 105:450-458

Mäkelä P, Kärkkäinen J, Somersalo S (2000) Effect of glycinebetaine on chloroplast ultrastructure, chlorophyll and protein content, and RuBPCO activities in tomato grown under drought or salinity. Biol Plant 43:471-475

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

Okuma E, Murakami Y, Shimoishi Y, Tada M, Murata Y (2004) Effects of exogenous application of proline and betaine on the growth of tobacco cultured cells under saline conditions. Soil Sci Plant Nutr 50:1301-1305

Okuma E, Soeda K, Tada M, Murata Y (2000) Exogenous proline mitigates the inhibition of growth of *Nicotiana tabacum* cultured cells under saline conditions. Soil Sci Plant Nutr 46:257-263

Pollard A, Wyn Jones RG (1979) Enzyme activities in concentrated solutions of glycinebetaine and other solutes. Planta 144:291-298

Rhodes D, Hanson AD (1993) Quaternary ammonium and tertiary sulfonium compounds in higher plants. Annu Rev Plant Physiol Plant Mol Biol 44:357-384

Roy D, Basu N, Bhunia A, Banerjee SK (1993) Counteraction of exogenous L-proline with NaCl in salt-sensitive cultivar of rice. Biol Plant 35:69-72

Shu QY, Liu GS, Xu SX, Li XF, Li HJ (2005) Genetic transformation of *Leymus chinensis* with the PAT gene through microprojectile bombardment to improve resistance to the herbicide Basta. Plant Cell Rep 24:36-44

Songstad DD, Duncan DR, Widholm JM (1990) Proline and polyamine involvement in chilling tolerance of maize suspension cultures. J Exp Bot 41:289-294

Zimmermann U (1978) Physics of turgor- and osmoregulation. Annu Rev Plant Physiol 29:121-148

Zobayed SMA, Saxena PK (2003) In vitro-grown roots: a superior explants for prolific shoot regeneration of St. John's wort (*Hypericum perforatum* L. cv 'New Stem') in a temporary immersion bioreactor. Plant Sci 165: 463-470