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Research Article

Changes in Antioxidant Enzyme Activity and Transcript Levels of Related Genes in *Limonium sinense* Kuntze Seedlings under NaCl Stress

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The halophyte *Limonium sinense* Kuntze is used in traditional Chinese medicine for clearing heat and for detoxification. To examine the detoxification and salt-tolerance mechanisms of this plant, we analyzed antioxidant enzyme activities and transcript levels of genes encoding antioxidant enzymes in *L. sinense* seedlings under salt stress (500 mmol/L NaCl). Catalase showed the largest increase in activity, peaking on day 4 of the 7-day NaCl treatment. Peroxidase and superoxide dismutase activities also increased, peaking on days 2 and 3 of the NaCl treatment, respectively. The activities of antioxidant enzymes decreased as the duration of the NaCl treatment extended. The transcript levels of genes encoding antioxidant enzymes were upregulated under NaCl stress. The peak in the *LsCAT* transcript level was earlier than the peaks in *LsAPX* and *LsGPX* transcript levels. The malondialdehyde content only slightly increased in *L. sinense* seedlings under NaCl stress. This was indicative of a low level of lipid peroxidation, consistent with the increased antioxidant enzyme activities and gene transcript levels. These results show that, under NaCl stress, the antioxidant system of *L. sinense* is activated and effectively scavenges reactive oxygen species. This reduces oxidative damage and allows the plant to maintain growth under NaCl stress.

1. Introduction

Salt in soils is the main environmental factor restricting the growth and productivity of agricultural crops [1]. Salt tolerance in plants is a complex process that involves changes in the structures of numerous tissues and organs and many physiological and biochemical processes [2]. In plants, salt stress results in increased generation of reactive oxygen species (ROS). These highly reactive substances can alter normal cellular metabolism via oxidative damage to membranes, proteins, and nucleic acids. They can also cause lipid peroxidation, denature proteins, and mutate DNA [3, 4].

Plants have developed a complex ROS-scavenging system to prevent sensitive cellular components from ROS-induced damage. This system comprises enzymatic and nonenzymatic antioxidants. The sum of the activities of all of the enzymes that make up the enzymatic antioxidant system represents the antioxidant capacity of plants. Catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) are important antioxidant enzymes that play key roles in eliminating superoxide (O_2^-) and hydrogen peroxide (H_2O_2).

Limonium sinense Kuntze is a perennial herb with strong tolerance to salt, aridity, and drought. This salt-secreting halophyte is used in traditional Chinese medicine for clearing heat and dampness, for hemostasis, and for detoxification. Its close relative, *Limonium gmelinii* (Wildl.) Kuntze, grows in salt meadows and has abundant salt glands on the adaxial and abaxial sides of the leaf [5]. The salt-tolerance mechanisms of *L. sinense* include its salt-secreting glands and its ability to compartmentalize ions in cells and regulate osmotic status. Early studies on this species focused on the structure of its salt glands and on methods for its tissue culture, rapid propagation, and artificial cultivation [6, 7]. Less attention has been paid to the physiological mechanisms of stress resistance in *L. sinense*. The aim of this study was to investigate ROS scavenging and the protective effects of the antioxidant

enzyme system in *L. sinense* seedlings by detecting changes in CAT, POD, and SOD activities and in the transcript levels of the genes encoding these enzymes, under NaCl stress.

2. Materials and Methods

2.1. Plant Material and Growth Conditions. Seeds of *L. sinense* were obtained from the halophyte garden of Dongying City, Shandong Province. Seeds were germinated in a growth chamber at 20°C. After 20 days of growth, seedlings were transplanted into soil in pots (5 seedlings/pot) and then grown in a greenhouse for 50 days under the following conditions: 12 h light/12 h dark photoperiod, 25°C, and 65% relative humidity.

2.2. Experimental Design. In each pot, seedlings were treated with 50 mL 500 mmol/L NaCl solution for 0, 1, 2, 3, 4, 5, 6, and 7 days. On each sampling day, the leaves of three seedlings in each group were harvested, weighed to determine fresh weight, frozen in liquid nitrogen, and then used for enzyme activity assays and gene expression analyses. All assays and measurements were conducted in triplicate.

2.3. Determination of Enzyme Activity and Isoenzyme Composition. The activities of CAT, POD, and SOD were assayed as described by Gao et al. [8]. Malondialdehyde (MDA) content was determined using the thiobarbituric acid reaction [9]. All values shown are the mean values of three assays. Differences between mean values of NaCl-treated samples and untreated controls were considered significant at $P < 0.05$. The isoform composition of CAT, POD, and SOD was determined as described by El-Mashad and Mohamed [10].

2.4. Determination of Gene Expression Level. RNA isolation, reverse transcription, and real-time quantitative PCR (qPCR) were conducted as described by Zhang et al. [11]. Sequence information was based on sequences in the *Limonium sinense* plasmid cDNA library. The qPCR primers used to amplify genes encoding catalase (*LsCAT*), ascorbate peroxidase (*LsAPX*), glutathione peroxidase (*LsGPX*), and actin (*LsActin*) were designed using Primers3 (http://bioinfo.ut.ee/primer3-0.4.0/). The sequences were as follows (F for forward, R for reverse).

The primers for *LsCAT* (BXC35):

F: 5′-CACATCCTCCTGTTGTTTCGGGTAG-3′;

R: 5′-ACAGGCTCAACGTCAGACCAACC-3′;

The primers for *LsAPX* (BXC1267):

F: 5′-TCCAAGGACCCTCAAAGCCAG-3′;

R: 5′-CCCTGACGCAAAGAAAGGAAATG-3′;

The primers for *LsGPX* (BXC0933):

F: 5′-ATGAAGGAAAGCAACGGGAAGAC-3′;

R: 5′-AGTAGCCAAGCCAAACAGATCAGAC-3′;

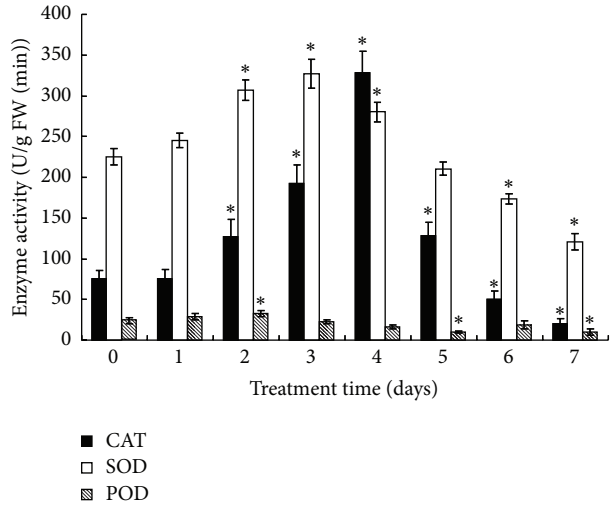

Figure 1: Activities of CAT, SOD, and POD in tissues of *L. sinense* under 500 mmol/L NaCl treatment. Values are means ± SD ($n = 3$). *Significant difference between untreated (day 0) and NaCl-treated sample ($P < 0.05$).

The primers for *LsActin*:

F: 5′-CAGTTTTCCCGAGTGTTGTTGGTAGG-3′;

R: 5′-ACCTCTTTTGGATTGTGCTTCGTCA-3′

To confirm specificity, primers and amplicons for each gene were compared with sequences in GenBank/NCBI. The gene *LsActin* was used as the reference gene. Relative expression of genes was calculated using the $2^{-\Delta\Delta Ct}$ method [12]. The experiments were repeated twice with three replicates.

2.5. Data Analysis. All data were analyzed using a one-way ANOVA with entries and sampling dates as factors. Mean separation processes were performed using a Fisher's protected LSD test at the $P < 0.05$ level of probability. For RT-qPCR, the $2^{-\Delta\Delta Ct}$ method is used to determine the changes of target gene expression based on normalization with the reference gene and one-way ANOVA was used to analyze differences between treatments' dates. The Pearson correlation analysis was made by SPSS software.

3. Results and Discussion

3.1. Antioxidant Enzymes' Activities and Isoenzymes of Seedlings. We measured the activities of antioxidant enzymes to evaluate the mechanisms of salt tolerance in *L. sinense*. The activity of CAT differed significantly between salt-stressed and untreated (day 0) seedlings. As shown in Figure 1, CAT activity showed the largest increase among all the antioxidant enzymes assayed. Compared with that on day 0, CAT activity increased by 0.7%, 68.7%, 154.6%, 336.2%, and 70.5% after 1, 2, 3, 4, and 5 days, respectively, of the 7-day NaCl treatment. The activity of CAT peaked on day 4 and then decreased as the duration of the NaCl treatment extended. The activity of SOD also showed a large increase under NaCl stress and peaked on

Figure 2: The phenotype of *L. sinense* seedlings under 500 mmol/L NaCl treatment.

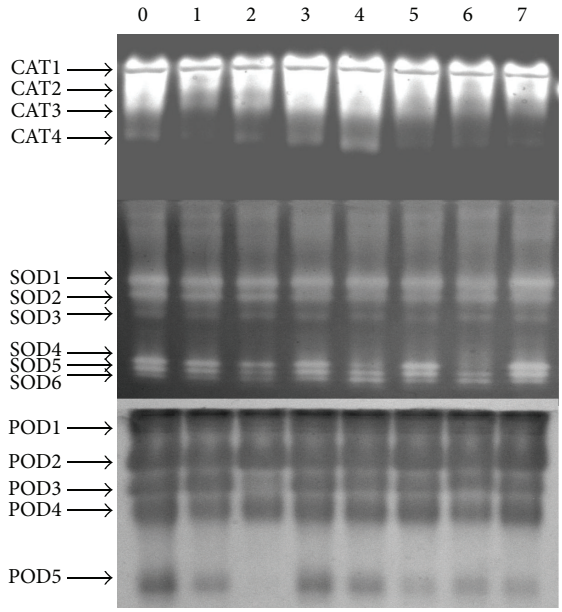

Figure 3: Identification of different isoenzymes (A-CAT, B-SOD, and C-POD) in *L. sinense* seedlings under NaCl stress. Each lane contains 45 μg protein. Arrows indicate different CAT, SOD, and POD isoenzymes.

day 3 of the 7-day salt treatment. Its activity decreased as the treatment time extended. Similarly, POD activity peaked on day 2 of the 7-day NaCl treatment (38.8% higher activity than that on day 0) and then decreased. The phenotype of *L. sinense* seedlings under NaCl stress was shown in Figure 2, which was in general accord with the activities of antioxidant enzymes.

As shown in Figure 3, the isoenzyme analysis revealed four CAT isoenzymes, six SOD isoenzymes, and five POD isoenzymes in *L. sinense*. The number of CAT, SOD, and POD isoenzymes changed under salt stress. The largest number of isoforms was on days 3-4 of the NaCl treatment for CAT, on day 2 for SOD, and on days 6-7 for POD. These results were consistent with the patterns of antioxidant enzyme activity during the NaCl treatment.

In plants, the activity of one or more antioxidant enzymes generally increases under stress conditions. This increase in antioxidant enzyme activity correlates with increased stress tolerance [13]. Antioxidant enzymes cooperate to remove excess ROS, thereby protecting the structures and functions of cellular components. SOD, which catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide, is an essential component of the antioxidant defense system in plants [14]. Several studies reported that SOD activity increased in response to excess salt in potato, switchgrass, and *Brassica juncea* [15–17], consistent with our results.

Our results suggested that the H_2O_2 produced via SOD activity was effectively consumed by CAT and/or POD. CAT, which is located in peroxisomes, catalyzes the dismutation of excess H_2O_2 into oxygen and water and maintains H_2O_2 at a low level. At low concentrations, H_2O_2 is mainly cleared by POD [14]. POD is widely distributed in plant tissues and is involved in diverse growth, development, and senescence processes in plants. Our findings suggested that the increased POD activity in *L. sinense* under salt stress might be sufficient to protect proteins and lipids against ROS. Induction of CAT and POD activities under salt stress has also been reported in tomato, sugar beet, and *Plantago* [18]. Our results showed that the activities of these antioxidant enzymes decreased as the duration of the salt stress treatment extended, even to levels below that in untreated samples. A similar result was reported for potato under salt stress [15].

3.2. Changes of Related Genes Expression of Seedlings. To investigate the molecular mechanism of salt tolerance in *L. sinense*, we used real-time qPCR to evaluate changes in the transcript levels of three genes: *LsCAT*, *LsAPX*, and *LsGPX*. The relative transcript levels of these three genes changed significantly under NaCl stress (Figure 4). After 3 days of NaCl stress, the transcript level of *LsCAT* was 100-fold than in the untreated control (Figure 4(a)). This gene was induced more strongly than the other two genes analyzed. The transcript levels of *LsAPX* also increased markedly under NaCl stress, to a maximum of 20.9 times than in the control after 5 days of NaCl stress. After 7 days of NaCl stress, the transcript level of *LsAPX* decreased to below the control level (Figure 4(b)). The transcript levels of *LsGPX* peaked at 6.13 times than in the control after 4 days of NaCl stress and then decreased to the control level or even lower as the duration of the NaCl treatment extended (Figure 4(c)).

To protect plant cells from oxidative injury caused by abiotic stresses such as salt stress, several genes encoding antioxidant enzymes including CAT, APX, and GPX are upregulated [19]. Therefore, analyses of the transcript levels of antioxidant defense genes can reflect the contributions of their products to the salt stress response in plants [20]. Our results showed that the peak in *LsCAT* transcript levels on day 3 was earlier than the peaks in the transcript levels of *LsAPX* and *LsGPX* (days 4-5). Additionally, the *LsCAT* transcript level was higher in NaCl-treated samples than in untreated samples throughout the treatment period. Induction of *CAT* gene expression under other abiotic stress conditions has been reported for other plant species [21]. We also observed that the upregulated transcript level of *LsCAT* was consistent

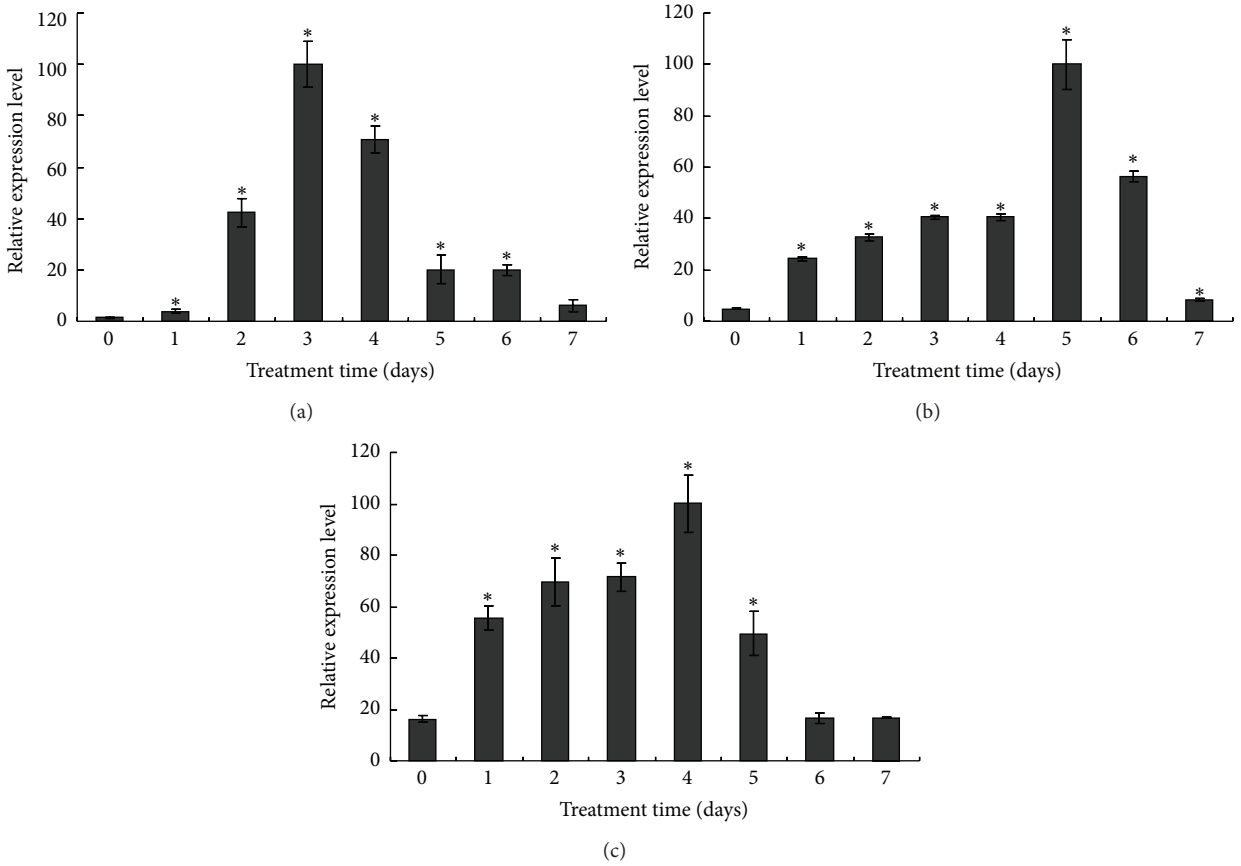

Figure 4: Relative transcript levels of *LsCAT* (a), *LsAPX* (b), and *LsGPX* (c) in *L. sinense* under 500 mM NaCl treatment. Transcript level of each gene was normalized to that of *LsActin*. Values are average relative expression level from three biological replicates. * Significant difference between NaCl-treated and untreated sample ($P < 0.05$).

with the increase in CAT enzyme activity, suggesting that CAT might play a key role in scavenging excessive ROS in NaCl-treated *L. sinense*.

Like CAT, APX has a high affinity for H_2O_2. In other studies, APX activity was shown to increase in response to a number of stress conditions, including salt stress [22, 23]. The transcript levels of *LsAPX* increased during the NaCl treatment, reaching peak levels on day 5 and then declining thereafter. Compared with transcription of *LsCAT*, that of *LsAPX* responded more slowly to salt stress. Similar results have been reported for other APX genes, for example, *Ec-apx1* from *Eleusine coracana*, in response to drought-induced oxidative stress [23].

The *LsGPX* transcript levels were higher in NaCl-treated seedlings than in untreated seedlings at most sampling times. Increased GPX activity could protect plant cells against the H_2O_2 released from the reaction catalyzed by SOD. In another study, it was reported that the transcript levels of *GPX* in *Panax ginseng* increased alongside an increase in GPX activity during abiotic stress [21].

3.3. MDA Content of Seedlings. The MDA content in leaves of *L. sinense* seedlings increased slightly throughout the duration of the NaCl treatment (Figure 5). The change in MDA content was not visible at the early stage (day 2), but after 3 days of salt treatment, it had increased to a level slightly higher than that in the control. The highest MDA content (twice that in the control) occurred after 6 days of NaCl stress. A significant negative correlation was observed between MDA content and SOD and POD activity ($R = -0.733$, $R = -0.791$, resp., $P < 0.05$), while no significant correlation was observed between MDA content and CAT activity ($R = -0.349$, $P > 0.05$).

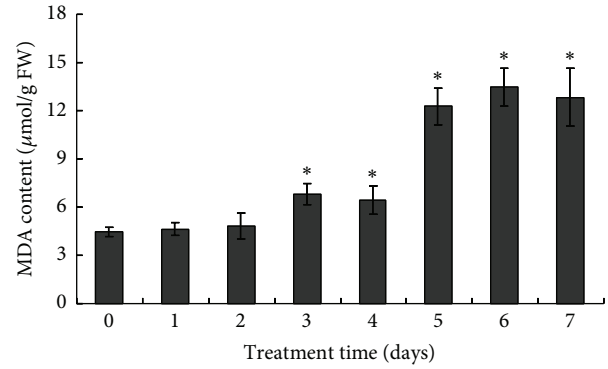

Figure 5: Effect of NaCl on MDA content of *L. sinense* seedlings. Values are means ± SD ($n = 3$). * Significant difference between NaCl-treated and untreated sample ($P < 0.05$).

Increased levels of lipid peroxides are indicative of enhanced ROS production [24]. MDA is a major cytotoxic product of lipid peroxidation, and therefore MDA content is another index to evaluate the extent of injury in plants under stress. It has been used as an indicator of free radical production in other studies [11]. The MDA content in seedlings of *L. sinense* only slightly increased under salt stress, suggesting that there was a low level of lipid peroxidation in these seedlings under salt stress. This might be because of the protective function of antioxidant enzymes in *L. sinense* seedlings, especially during the early phase of the salt stress response. These results indicate that the synergistic effect of various ROS-scavenging enzymes can effectively improve the antioxidant capacity of plants, thereby reducing the degree of oxidative injury.

4. Conclusion

The findings of this study indicate that the ability of *L. sinense* to cope with salt stress depends on antioxidant stress defense mechanisms. An increase in antioxidant enzyme activity and the resulting increase in ROS-scavenging capacity can improve the salt tolerance of plants [25]. This relationship between salt tolerance and increased activities of antioxidant enzymes has been demonstrated in pea [26], *Arabidopsis*, and rice [27]. Our results show that there were increases in antioxidant enzyme activity and in the transcript levels of genes encoding these enzymes under salt stress. These findings suggest that the response of *L. sinense* to salt stress involves CAT, SOD, APX, POD, and GPX. These results increase our understanding of how *L. sinense* responds to salt stress and provide evidence for the effectiveness of its detoxification mechanisms. At present, we are cloning and functionally analyzing *CAT* and *GPX* genes of *L. sinense* to investigate their regulation patterns in this salt-tolerant plant.

Conflict of Interests

The authors declare that they have no financial and personal relationships with other people or organizations that can inappropriately influence their work; there is no professional or other personal interests of any nature or kind in any product, service, and/or company that could be construed as influencing the position presented in, or the review of, this paper.

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