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Effects of glycine pretreatment on the growth and oxidative damage in heat-stressed *Festuca sinensis* Keng seedlings

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Abstract: The protective effects of glycine on alleviating the heat stress of *Festuca sinensis* Keng seedlings were investigated. It was found that glycine pretreatment reduced the reactive oxygen species (ROS) level and protected the plasma membrane integrity of seedlings under heat stress, thus reducing cell death of the roots exposed to high temperature stress. Further study indicated that glycine pretreatment improved the activities of superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) in the seedlings. Semi-quantitative RT-PCR analysis indicated that the transcripts of CAT gene in glycine pretreated-seedlings were higher compared to untreated control seedlings under heat stress. The involvement of antioxidative enzymes activities in glycine-mitigated heat stress was also discussed.

Key words: *Festuca sinensis* Keng; glycine; heat stress; antioxidative enzyme

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甘氨酸预处理对中华羊茅热胁迫下幼苗生长和氧化损伤的影响

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摘要: 研究了甘氨酸预处理对降低中华羊茅幼苗热胁迫的保护性作用。甘氨酸预处理降低了活性氧水平, 保护了热胁迫下幼苗细胞膜的完整性, 因而降低了植株根系细胞的死亡率。研究表明: 甘氨酸预处理可以提高幼苗超氧化物歧化酶(SOD), 过氧化氢酶(CAT)和抗坏血酸过氧化物酶(APX)的活性。半定量RT-PCR分析表明甘氨酸预处理幼苗中CAT基因的表达比对照高。讨论了抗氧化酶活性在甘氨酸调节热胁迫中的作用。

关键词: 中华羊茅; 甘氨酸; 热胁迫; 抗氧化酶

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Abiotic stresses, such as drought, salinity, high temperature, chemical toxicity and oxidative stress, inhibit the growth and development of plants by disturbing many biochemical and physiological

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processes^[1]. High temperature stress, an important environmental factor in the northern regions of the China, seriously effects forage growth and development, and leads to a reduction of forage primary productivity^[2]. High temperature induces reactive oxygen species(ROS) accumulation and can directly or indirectly inhibit a series of physiological processes and even cause plant death. These processes contain photosynthesis, plant-water relationships, cell elongation and nitrogen metabolism.

Plant growth and development are closely associated with leaf nitrogen nutrition^[3-4]. It is believed that exogenous amino acids, such as glycine, could be utilized as an organic nitrogen source in plants, and participates in photosynthesis and carbohydrate metabolism^[5-6]. However, the effects of glycine on abiotic stress tolerance are not fully understood.

Reactive oxygen in species cause deterioration of membrane function^[7]. Heat stress induces the over-accumulation of reactive oxygen species such as hydrogen peroxide (H_2O_2) and hydroxyl radicals, resulting in lipid peroxidation. To avoid the deleterious effects of ROS, plant cells possess efficient antioxidative enzymes, such as superoxide dismutase(SOD), catalase(CAT) and ascorbate peroxidase(APX), etc. These antioxidative enzymes have been observed to specifically counteract oxidative damage in plants subject to heat stress.

Festuca sinensis Keng is a dominant plant species of the grassland. It is mainly distributed in the northwest, north, northeast and the Qinghai-Tibetan plateau of China. In the present study, we focused on the effects of glycine pretreatment on the heat stress in *Festuca sinensis* Keng. The involvement of antioxidative enzymes activities in glycine-mitigated heat stress was also discussed.

1 Materials and methods

1.1 Plant material and culture conditions

Seeds of the *Festuca sinensis* Keng were first treated with 70% ethanol for 30 s and then immersed in 1% (*w/v*) sodium hypochlorite for 8 min, rinsed five times with sterile distilled water, and then left to germinate on Petri dishes with double-layer filter paper wetted with distilled water. Seven-day-old

Festuca sinensis Keng seedlings were transferred to flasks with half-strength Hoagland solution for three weeks. To study the effect of glycine pretreatment on the growth of seedlings, three-old-week seedlings were pretreated with 300 $\mu\text{mol/L}$ glycine for 4 h, and then transferred to half-strength Hoagland solution under various temperature conditions (25 °C, 35 °C).

1.2 Determination of ROS level and cell death in the root tips

To measure the levels of ROS in the roots of *Festuca sinensis* Keng seedlings, roots were stained with 25 $\mu\text{mol/L}$ 5-(and 6-)chloromethyl-2', 7'- dichlorodihydro-fluorescein diacetate, acetylerster (CM-H2DCFDA; Molecular Probes, USA) for 15 min. The roots were then washed with distilled water for three times and viewed under a confocal laser-scanning microscope (LSM510; Zeiss). All of the images were scanned using the same conditions.

To measure the level of cell death in the root tips, we incubated the roots with 3 $\mu\text{g/mL}$ propidium iodide(PI) dissolved in distilled water for 4 min. For each treatment, at least 20 seedlings were analyzed.

1.3 Determination of H_2O_2 level in the leaves

Leave segments were immersed in 1 mg/mL of 3-diaminobenzidine (DAB)-HCl (pH 3.8) and placed under the light for 5 h and then boiled in alcohol (95%, *v/v*) for 5 min. Photos were taken using a Carl Zeiss imaging system. Five leave segments were analyzed in each set of experiments.

2 Enzyme activity

Fresh leaves (0.5 g) were homogenized in 50 mmol/L of naphosphate buffer (pH 7.8) containing 0.1 mmol/L of EDTA and 1% (*w/v*) of polyvinylpyrrolidone at 4 °C. The homogenate was centrifuged at 10 000 g for 15 min at 4 °C. The supernatant was used to measure the activities of enzymes. The activity of SOD was measured as described by Beauchamp et al^[8]. The activity of CAT was determined using guaiacol and H_2O_2 substrates, as described previously^[9]. The activity of APX was measured as described by Nakano et al^[10].

3 Semi-quantitative RT-PCR analysis

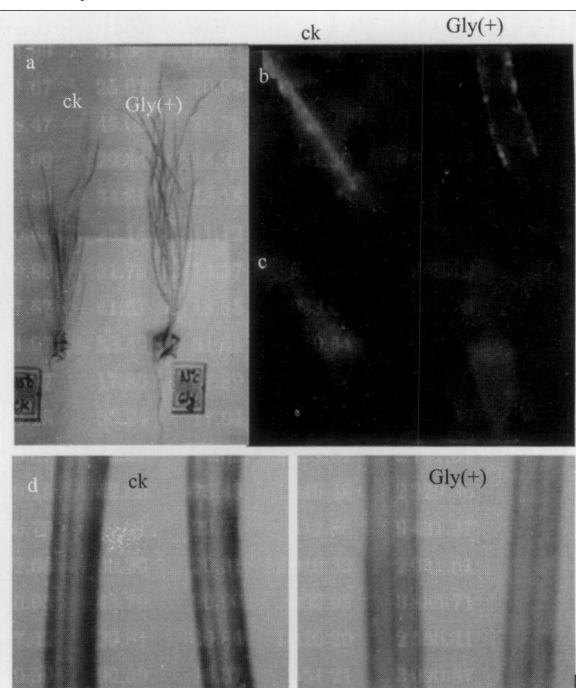
The total RNA was isolated from the seedlings using Trizol (Gibco/Brl, life technologies, Gaithersburg, MD, USA). For the semi-quantitative RT-PCR reaction, we quantified the concentration of RNA accurately using spectrophotometry. The cDNA was synthesized from DNase-treated total RNA using the reverse transcription system kit (Promega, Madison, WI, USA) and oligo-dTprimers. We performed control reactions using the 18SrRNA primers (5'-CCTTCGTCTTGA TCTTGCGG-3' and 5'-AGCGATGGCTGGAACAGAAC-3') to ensure that equal amounts of RNA were used in each set of reactions. The primers used to amplify CAT gene were as follows: FaCAT1 (ALW380120): 5'-ATC TTC TCC TAC TCC GAC AC-3' and 5'-AAA GGT ACT TTC AGC ATC GG-3'.

4 Results and discussion

As shown in Figure 1a, glycine pretreatment improved heat stress tolerance in *Festuca sinensis* seedlings. High-temperature-induced oxidative damage is an important factor leading to cell death in root tips, and thereby inhibiting the seedling growth. Pretreatment with glycine reduced the accumulation of ROS in roots (Figure 1b) and therefore improved the integrity of the plasma membrane in roots (Figure 1c). PI is a membrane-impermeable dye that binds to nucleotides and is generally excluded from living cells. Labeling of the nucleus of cells is a strong indication of a loss of membrane integrity^[11]. Staining with PI showed that glycine improved the cell survival and protected the integrity of the plasma membrane in the roots of seedlings.

Leaves are more sensitive to changes in temperature than roots. When coming across heat stress, leaves would be harmed prior to roots. H₂O₂ is a kind of ROS. The level of H₂O₂ can mainly reflect the membrane injury in leaves. To examine the accumulation of H₂O₂ under high temperature in *Festuca sinensis* Keng leaves, we applied DAB staining to detect H₂O₂ level in vivo. As shown in Figure 1d, high temperature stress increased the accumulation of H₂O₂ in leaves. Pretreatment with glycine markedly reduced the level of H₂O₂ in leaves. To

a certain extent, it protected the integrity of the plasma membrane in the leaves as well as roots of seedlings.



a Effects of glycine pretreatment on growth of *Festuca sinensis* Keng seedlings. In situ fluorescence and histochemical localization showing the effects of glycine pretreatment on high temperature-induced; b ROS accumulation in roots; c Loss of membrane integrity in roots; d H₂O₂ level in leaves in *Festuca sinensis* Keng seedlings

Figure 1 Glycine pretreatment alleviates heat stress in *Festuca sinensis* Keng seedlings

From these results, we can come to the conclusion that pretreatment with glycine can enhance the antioxidative capacity in *Festuca sinensis* Keng seedlings. To elucidate the effects of glycine pretreatment on antioxidative capacity, we examined the activities of antioxidative enzymes in seedlings. Pretreatment with glycine increased the activities of SOD, CAT and APX by 20%, 23.8% and 21.7%, respectively, compared to untreated control after 12 h exposure to high temperature stress (Figure 2), indicating that glycine pretreatment repressed ROS production by increasing the activities of antioxidative enzymes. However, as the treated time went on, whether for untreated control or for glycine-pretreated seedlings, only the activity of SOD increased; CAT and APX tended to go down. This indicated that SOD may be more related to heat tolerance. Although the activities of CAT and APX de-

creased, compared to untreated control, the decrease is rather small. In this aspect, they still contributed to the antioxidative capacity under heat stress.

The above studies showed that pretreatment with glycine improved heat stress tolerance in *Festuca sinensis* Keng seedlings by increasing the activities of antioxidative enzymes. We then examined the ex-

pression of CAT in *Festuca sinensis* Keng seedlings. As shown in Figure 2d, glycine pretreatment up-regulated the expression of CAT in *Festuca sinensis* Keng seedlings exposed to high temperature. These results indicated that glycine modulated the gene expression of antioxidative enzymes under heat stress, thus promoting the seedling growth.

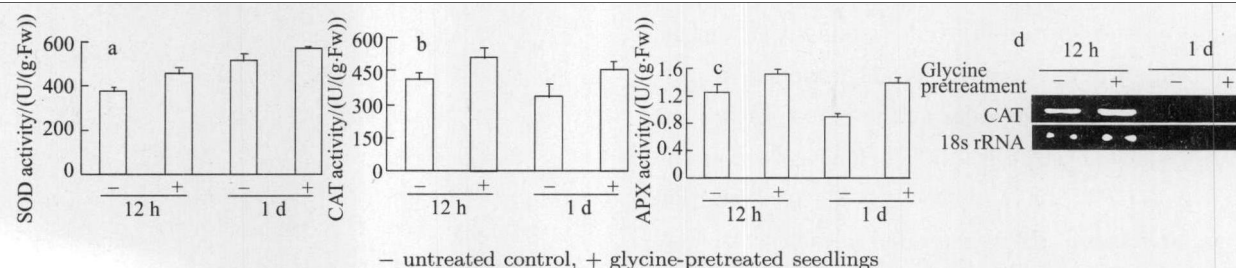


Figure 2 Effects of glycine pretreatment on the activities of SOD, CAT, APX and the gene expression of CAT in *Festuca sinensis* Keng seedlings

In this work, we provide evidence for the involvement of glycine in high temperature tolerance. Glycine reduced ROS accumulation by increasing the gene expression and activity of antioxidative enzyme and improving plasma membrane integrity, thereby promoting seedling growth under high temperature stress. These results suggested that elevated glycine levels could improve the detoxification capacity in plants. The study is useful for further elucidating the effects of glycine on plant growth under heat stress and these understandings could help us improve pasture heat tolerance and yield by using the combinatorial approaches of biotechnology and management.

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