

Salicylic Acid Decreases the Levels of Dehydrin-Like Proteins in Tibetan Hulless Barley Leaves under Water Stress

Xin Sun, Shu Yuan, and Hong-Hui Lin*

Key Laboratory of Bio-resources and Eco-environment (Ministry of Education), College of Life Science, Sichuan University, Chengdu 610064, Sichuan, P. R. China.

Fax: 86-028-8541 25 71. E-mail: honghuilin@hotmail.com

* Author for correspondence and reprint requests

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The effects of salicylic acid (SA) on the accumulation of dehydrins in leaves of Tibetan hulless barley seedlings under water stress were investigated. The results indicated that SA decreased the levels of the four dehydrin-like proteins induced by water stress. The concentrations of these dehydrin-like proteins increased under water stress. However, their levels in SA-pretreated seedlings were always lower than in those receiving only water stress. Our results also indicated that the levels of dehydrin-like proteins decreased as the SA concentration increased. In SA-pretreated seedlings, electrolyte leakage, MDA and H₂O₂ content were rather higher than in seedlings receiving only water stress. By these results, we suggest that lower levels of dehydrin-like proteins in seedlings with SA treatment may be due to the greater accumulation of H₂O₂ induced by SA, which causes more oxidative injury under water stress.

Key words: Salicylic Acid (SA), Dehydrin, Water Stress

Introduction

Dehydrins, which are known as late embryogenesis abundant (LEA) D-11 protein family, usually accumulate in plants during late embryogenesis or in response to environmental stresses causing cell dehydration (Close, 1996), such as low temperatures (Borovskii *et al.*, 2002; Stupnikova *et al.*, 2002), drought (Labhili *et al.*, 1995), and salinity (Godoy *et al.*, 1994). Many studies indicated that dehydrins are associated with specific protective functions under conditions of cell dehydration. They may prevent coagulation of macromolecules and maintain integrity of crucial cell structures like plasma membranes (Campbell and Close, 1997). Many dehydrins have been identified in response to abscisic acid (ABA) as a regulator. Expression of these dehydrin genes has been proved to be regulated by ABA (Allagulova *et al.*, 2003). But there are few data which show the relationship between the expression of dehydrins and other bioregulators.

It is known that salicylic acid (SA) is an important bioregulator in plants. It can activate gene expression and influence a variety of signaling mechanisms in plant defense (Shah, 2003). SA plays an important role in the defense response to environmental stresses in many plant species (Senaratna *et al.*, 2000; Nemeth *et al.*, 2002). So, the effects of

SA on the accumulation of dehydrins in plants are worth to be studied. However, there is few data showing the relationship between SA and dehydrins. In the present study, we investigated the effects of SA on oxidative damage and accumulation of dehydrins in leaves of Tibetan hulless barley seedlings. The possible reasons for the changes in dehydrin levels are also discussed.

Materials and Methods

Plant growth and treatments

Sterilized seeds of Tibetan hulless barley (*Hordeum vulgare* L. var. *nudum* Hook. f.) were germinated on water-moistened filter paper for 2 d in the dark at 25 °C, then grown in ½ Hoagland's solution at 22/20 °C (day/night) with a 14 h light/10 h dark photo-cycle in the green house at a light intensity of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$. For water stress, fifteen-day-old seedlings were transferred to PEG-6000 solutions with an osmotic potential of –0.5 MPa for 24, 48, and 72 h, respectively. Before water stress, part of the seedlings were treated with 0.5, 1.0, 1.5 mM SA, respectively, for 24 h.

Protein extraction

Approx. 1 g of fresh leaves was cut into small pieces and homogenized with 5 ml of extraction

buffer containing 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.5, 20 mM NaCl and 1 mM phenylmethylsulphonyl fluoride (PMSF) in an ice bath. The resulting slurry was transferred into a tube and centrifuged at $10,000 \times g$ for 10 min at 4 °C. The supernatant was transferred to a new tube and stored at -80 °C. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

Protein electrophoresis and western blot analysis

For SDS-PAGE and western blot analysis, 10 μ g of protein from each sample were electrophoresed in 15% polyacrylamid gels and then transferred electrophoretically to a nitrocellulose membrane according to Sambrook *et al.* (1989). After transfer, the nitrocellulose membrane was blocked with 5% nonfat milk in TBS (Tris-buffered saline) for 2 h and then incubated with primary antibody (rabbit anti-dehydrin) for 2 h. After washing with TBS for three times and TTBS (TBS with 0.05% Tween-20) once, the nitrocellulose membrane was incubated with goat anti-rabbit IgG alkaline phosphatase conjugate (dilution 1:500) for 1 h. After washing with TBS, the secondary antibody was detected using nitroblue-tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP).

Electrolyte leakage measurements

The electrolyte leakage of leaves was measured according to Nanjo *et al.* (1999) with some modification. Approx. 0.2 g of fresh leaves were cut into about 1 cm pieces and placed in 5 ml deionized water at room temperature. After 45 min, the conductivity (C_1) was measured and then the samples were incubated in a boiling water bath for 10 min to achieve 100% electrolyte leakage (C_2). The results were calculated according to the formula $(C_1/C_2) \times 100\%$.

Malondialdehyde (MDA) contents measurements

The MDA content of leaves was measured by the method of Karabal *et al.* (2003) with some modification. Approx. 0.2 g of fresh leaves were cut into small pieces and homogenized by the addition of 5 ml 5% trichloroacetic acid (TCA) in an ice bath. The homogenate was transferred into a tube and centrifuged at $1,000 \times g$ for 10 min at 4 °C. Aliquots of supernatant and 0.5% thiobarbituric acid (TBA) in 20% TCA solution were added

into a new tube. This mixture was incubated at 98 °C for 40 min, then cooled to room temperature and centrifuged at $8,000 \times g$ for 5 min. The supernatant was subjected to analysis with the spectrophotometer. The MDA content was calculated from the subtracted absorbance ($A_{535} - A_{600}$) using the extinction coefficient of $155 \text{ mm}^{-1} \text{ cm}^{-1}$.

Hydrogen peroxide (H_2O_2) contents measurements

The H_2O_2 content of leaves was measured as described by Velikova *et al.* (2002). Approx. 0.5 g of fresh leaves were cut into small pieces and homogenized in an ice bath with 5 ml 0.1% (w/v) TCA. The homogenate was transferred into a tube and centrifuged at $12,000 \times g$ for 20 min at 4 °C. 0.5 ml of the supernatant was added to 0.5 ml 10 mM potassium phosphate buffer (pH 7.0) and 1 ml 1 M KI. The absorbance of supernatant was read at 390 nm. The content of H_2O_2 was determined by a standard curve.

Results

Dehydrin expression in leaves under water stress and SA treatment

After seedlings were grown in PEG solution for 24 h, four dehydrin-like proteins were detected by western blot analysis. The molecular masses of the detected proteins were around 16, 20, 27, and 78 kDa (Fig. 1A). But in the control seedlings (without PEG treatment), no dehydrin-like protein was detected. The same four dehydrin-like proteins were also detected in the seedlings pretreated with 0.5 mM SA and water stress for 24 h. But the levels were lower than those in seedlings only treated with PEG for the same time. No dehydrin-like protein was detected in the seedlings pretreated with 0.5 mM SA followed by no PEG treatment (Fig. 1A).

During 72 h of water stress, levels of the four dehydrin-like proteins increased more as the seedlings were held longer in PEG. Accumulation of these proteins also increased in the seedlings receiving pretreatment with 0.5 mM SA. But in contrast to those in seedlings treated with PEG alone, the levels were obviously lower (Fig. 1B).

In order to test the effects of SA concentration on the expression of dehydrin, we used three different concentrations of SA (0.5 mM, 1.0 mM, 1.5 mM) to treat the seedlings before water stress. Higher levels of the four dehydrin-like proteins were detected in the seedlings pretreated with

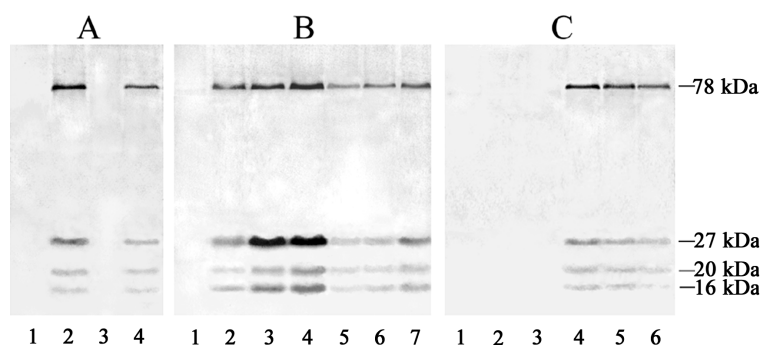


Fig. 1. The accumulation of dehydrin-like proteins in leaves of Tibetan hulless barley seedlings. (A) Western blot analysis of dehydrin-like proteins induced by different treatments: lane 1, untreated control; lane 2, PEG treatment for 24 h alone; lane 3, 0.5 mM SA pretreatment followed by no PEG treatment; lane 4, 0.5 mM SA pretreatment followed by 24 h of PEG treatment. (B) Western blot analysis of dehydrin-like proteins during 72 h of PEG treatment after 0.5 mM SA pretreatment or no SA pretreatment: lane 1, untreated control; lane 2, PEG treatment for 24 h alone; lane 3, PEG treatment for 48 h alone; lane 4, PEG treatment for 72 h alone; lane 5, SA pretreatment followed by 24 h of PEG treatment; lane 6, SA pretreatment followed by 48 h of PEG treatment; lane 7, SA pretreatment followed by 72 h of PEG treatment. (C) Western blot analysis of dehydrin-like proteins under different concentrations of SA followed by no PEG treatment or 24 h of PEG treatment: lane 1, 0.5 mM SA pretreatment followed by no PEG treatment; lane 2, 1.0 mM SA pretreatment followed by no PEG treatment; lane 3, 1.5 mM SA pretreatment followed by no PEG treatment; lane 4, 0.5 mM SA pretreatment followed by 24 h of PEG treatment; lane 5, 1.0 mM SA pretreatment followed by 24 h of PEG treatment; lane 6, 1.5 mM SA pretreatment followed by 24 h of PEG treatment. Mass (kDa) of proteins is indicated on the right.

0.5 mM SA rather than in those pretreated with 1.0 mM or 1.5 mM SA. It could be clearly seen that the high concentration of SA prevented the accumulation of the four dehydrin-like proteins more than low concentrations. However, no dehydrin-like protein, like the situation in the seedlings treated with 0.5 mM SA, was detected in the seedlings pretreated with 1.0 mM and 1.5 mM SA followed by no PEG treatment (Fig. 1C).

Effects of water stress and SA on electrolyte leakage of leaves

Electrolyte leakage reflects the changes of cell membrane structure under water stress. Its relative conductivity can be used to evaluate the damage on structure and function of cell membranes. In order to test the effects of SA on electrolyte leakage of leaves under water stress, seedlings were treated with 0.5 mM SA before water stress. Results showed that the electrolyte leakage of seedlings receiving pretreatment with 0.5 mM SA, in contrast to that only treated with PEG, increased remarkably. Although the slight increase at the beginning (0 h) was not statistically significant, a significant difference could be observed between the two groups after 24 h of water stress (Fig. 2). These results suggested that the cell membrane structure of Tibetan hulless barley leaves re-

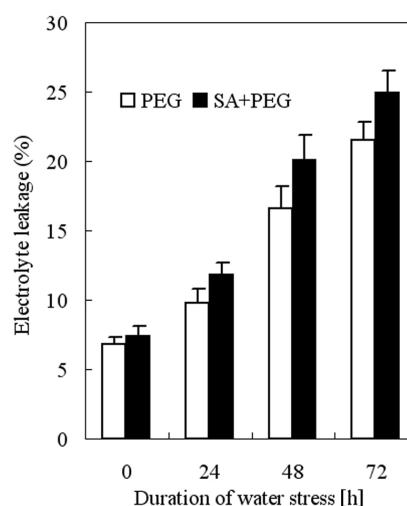


Fig. 2. Water stress and SA on electrolyte leakage of Tibetan hulless barley seedling leaves. Bars represent standard deviations of 5 independent replicates.

ceived more damage after pretreatment with 0.5 mM SA.

Effects of water stress and SA on MDA content of leaves

The MDA content was measured to determinate the extent of lipid peroxidation. The treatment of

testing electrolyte leakage was repeated. Over the whole range of water stress, the MDA content of seedlings with SA pretreatment was higher than that in leaves without SA pretreatment, although the difference at the beginning of water stress (0 h) was not very significant (Fig. 3). These results reflected that SA caused more lipid peroxidation in Tibetan hulless barley leaves under water stress.

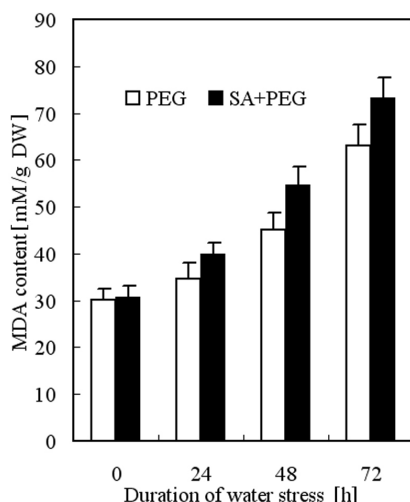


Fig. 3. Water stress and SA on MDA content of Tibetan hulless barley seedling leaves. Bars represent standard deviations of 5 independent replicates.

Effects of water stress and SA on H₂O₂ content of leaves

In order to determine the changes in H₂O₂ content, the same treatment in testing electrolyte leakage and MDA content was applied. Higher content of H₂O₂ was always detected in SA-pretreated seedlings rather than no SA-pretreated seedlings during the same time of water stress (Fig. 4). This suggested that SA enhanced the accumulation of H₂O₂ in leaves of seedlings under water stress.

Discussion

Gene expression and protein accumulation of dehydrins have been observed in plants exposed to environmental abiotic stresses, such as drought, cold, and salinity, which have a dehydration component (Close, 1997). These environmental stresses are always related to the formation of radicals such as superoxide radicals (O₂[•]), hydrogen peroxide (H₂O₂) and hydroxyl radicals (•OH) in

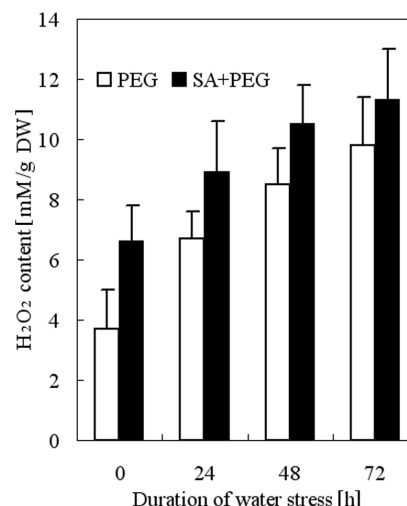


Fig. 4. Water stress and SA on H₂O₂ content of Tibetan hulless barley seedling leaves. Bars represent standard deviations of 5 independent replicates.

plants. These radicals can damage many cellular components including proteins, membrane lipids and nucleic acids. In plants, superoxide radicals can be converted to H₂O₂ by superoxide dismutase (SOD) and then reduced or used by catalase (CAT) and other peroxidases. Hydroxyl radicals are generated mainly in Fenton-type Haber-Weiss reactions which require catalytic metals and H₂O₂ (Jiang, 1999). These processes are vital for protecting cells against oxidative injury (Mittler, 2002).

In the present study, higher electrolyte leakage, MDA content, and H₂O₂ levels could be observed in Tibetan hulless barley seedling leaves receiving pretreatment with SA before water stress than in seedlings without SA treatment. Our results suggest that the SA-pretreated seedlings suffered more membrane damage and oxidative stress. This is in good agreement with experiments demonstrating that 0.5 mM SA caused increased sensitivities to drought in both maize and wheat (Nemeth *et al.*, 2002). Previous studies showed that SA caused inhibition of CAT activity (Sanchez-Casas and Klessig, 1994) and enhanced H₂O₂ levels (Rao *et al.*, 1997), and therefore, resulted in heavy lipid peroxidation and oxidative damage (Rao *et al.*, 1997). According to the results of Borsani *et al.* (2001), transgenic *Arabidopsis* plants (*NahG*) producing salicylate hydroxylase, which transforms SA to catechol, suffered less oxidative damage generated by salt and osmotic stress than wild-type plants. This result also

indicated that SA increases the oxidative injury of plants under stress.

On the other hand, the concentrations of dehydrin-like proteins in leaves of seedlings increased during 72 h of water stress. For the same time, however, lower levels of dehydrin-like proteins were detected in seedlings receiving pretreatment with SA. Dehydrins were demonstrated to have radical scavenging activity and inhibitory activity against lipid peroxidation. Dehydrins like CuCOR19 could be oxidatively modified by radicals like hydroxyl radicals and peroxy radicals, so they could deplete radicals and reduce oxidative damages induced by water stress (Hara *et al.*, 2004). But exceeding hydroxyl radicals induced by a high level of H₂O₂ may cause great degradation of dehydrins, and result in low levels of dehydrins (Hara *et al.*, 2004). It is indicated that the concentration of catalytic metals increased under water stress (Jiang, 1999), and the concentration of H₂O₂ was higher after SA treatment. Therefore, there are possibly more hydroxyl radicals generated by the Fenton-type Haber-Weiss reactions in water stressed plants. We suggest that higher content of H₂O₂ after SA treatment may induce more hydroxyl radicals under water stress. And the low levels of dehydrin-like proteins in SA-pretreated seedlings may attribute to their degradation caused by exceeding radicals like hydroxyl radicals. At least, an enhanced H₂O₂ level induced by SA was

one of the important reasons for declines of dehydrins.

For further investigations, we tested the effects of different SA concentrations (0.5 mM, 1.0 mM, and 1.5 mM) on the protein levels of dehydrins in leaves of Tibetan hulless barley seedlings. As a result, the levels of the four detected dehydrin-like proteins decreased along with the SA concentration increased. Recently, Shen *et al.* (2004) studied the expression of *BcDh2* gene (a novel dehydrin-like gene from *Boea crassifolia*) in response to SA. Their northern analysis of *BcDh2* mRNA showed that more transcripts were detected at lower concentration of SA rather than at higher concentration. These results all suggested that higher concentration of SA enhanced the inhibition of dehydrin accumulation.

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