Journal of Plant Physiology 168 (2011) 1562–1567

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

Hydrogen peroxide spraying alleviates drought stress in soybean plants

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ARTICLE INFO

Article history:
Received 29 October 2010
Received in revised form 10 February 2011
Accepted 10 February 2011

Keywords:
Glycine max
Hydrogen peroxide
Leaf relative water content
Myo-inositol and galactinol metabolism
Water stress

ABSTRACT

To ascertain the effect of exogenously applied hydrogen peroxide (H_2O_2) on drought stress, we examined whether the spraying of soybean leaves with H_2O_2 would alleviate the symptoms of drought stress. Pre-treatment by spraying leaves with H_2O_2 delayed foliar wilting caused by drought stress compared to leaves sprayed with distilled water (DW). Additionally, the relative water content of drought-stressed leaves pre-treated with H_2O_2 was higher than that of leaves pre-treated with DW. Therefore, we analyzed the effect of H_2O_2 spraying on photosynthetic parameters and on the biosynthesis of oligosaccharides related to water retention in leaves during drought stress. Under conditions of drought stress, the net photosynthetic rate and stomatal conductance of leaves pre-treated with H_2O_2 were higher than those of leaves pre-treated with DW. In contrast to DW spraying, H_2O_2 spraying immediately caused an increase in the mRNA levels of D-*myo-inositol 3-phosphate synthase* 2 (*GmMIPS*2) and *galactinol synthase* (*GolS*), which encode key enzymes for the biosynthesis of oligosaccharides known to help plants tolerate drought stress. In addition, the levels of *myo*-inositol and galactinol were higher in H_2O_2-treated leaves than in DW-treated leaves. These results indicated that H_2O_2 spraying enabled the soybean plant to avoid drought stress through the maintenance of leaf water content, and that this water retention was caused by the promotion of oligosaccharide biosynthesis rather than by rapid stomatal closure.

Introduction

Plants respond and adapt to water deficits at both the cellular and the molecular levels by the accumulation of osmolytes and proteins that are specifically involved in stress tolerance. An assortment of genes with diverse functions are induced or repressed by drought stress (Shinozaki et al., 2003; Bartels and Sunkar, 2005; Yamaguchi-Shinozaki and Shinozaki, 2005). Furthermore, the physiological responses induced by molecular signaling under conditions of drought stress include stomatal closure, the repression of cell growth and photosynthesis, and the activation of respiration. Consequently, drought stress is the primary cause of crop loss across the globe, reducing average yields in most major crop plants (Boyer, 1982; Bray et al., 2000).

Soybean yield is highly affected by soil water supply (Ashley and Ethridge, 1978). In soybeans, drought stress occurring at the early stage of pod development significantly increases the rate of pod abortion, thus decreasing final seed yield (Liu et al., 2003). Saitoh et al. (1999) reported that from the late-flowering stage to the young-pod stage, the soybean is susceptible to sink abortion, and the seed growth stage at the time of stress is the most critical factor determining the degree of yield loss under drought stress. Brevedan and Egli (2003) also reported that short periods of water stress during seed filling caused substantial yield reduction due to the presence of fewer and smaller seeds in the soybean. Therefore, there is an urgent need to better understand the impact of drought on plant function, and particularly on the physiological mechanisms underlying plant responses during and after drought stress.

It is known that drought stress enhances the production of reactive oxygen species (ROS) in cellular compartments such as chloroplasts, peroxisomes, and mitochondria. It is generally accepted that the imposition of an environmental stress, such as drought, chilling, heat, or high-light irradiation, gives rise to high concentrations of ROS such as superoxide, H_2O_2, singlet oxygen, and hydroxyl radical (Bowler et al., 1992; Foyer et al., 1994; Alscher et al., 1997; Shigeoka et al., 2002). If drought stress is pro-

Abbreviations: DAT, days after treatment; DW, distilled water; *E*, transpiration rate; GolS, galactinol synthase; g_s, stomatal conductance; H_2O_2, hydrogen peroxide; MIPS, D-*myo*-inositol 3-phosphate synthase; P_N, net photosynthetic rate; SWC, soil water content; RFOs, raffinose-family oligosaccharides; ROS, reactive oxygen species; RWC, leaf relative water content.

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doi:10.1016/j.jplph.2011.02.003

longed, ROS production will overwhelm the scavenging action of the antioxidant system, resulting in extensive cellular damage and death (Cruz de Carvalho, 2008). On the other hand, ROS are also known to function as signal molecules in plants (Foyer et al., 1997), controllingprocesses such as growth, development, responses to biotic and abiotic environmental stimuli, and programmed cell death (Bailey-Serres and Mittler, 2006).

Abscisic acid (ABA), synthesized in response to drought stress, is known to induce stomatal closure and to reduce transpirational water loss (Schroeder et al., 2001). ABA activates the synthesis of ROS in guard cells by a membrane-bound NADPH oxidase, and ROS mediate stomatal closure by activating (through hyperpolarization) plasma membrane Ca^{2+} channels (Pei et al., 2000; Murata et al., 2001; Wang and Song, 2008). In addition, it has been reported that hydrogen peroxide (H_2O_2), a type of ROS, is involved in the acclimation of *Cistus albidus* to summer drought (Jubany-Marí et al., 2009) and in that of maize (*Zea mays* L.) to salt stress (Azevedo Neto et al., 2005). H_2O_2 also increases the soluble sugar content of melon fruits (Ozaki et al., 2009). Other components may also be responsive to ROS as a part of a stress-activated signal transduction pathway. We therefore focused on ROS as signal molecules by examining whether exogenous H_2O_2 application (by spraying) could alleviate drought stress and by working to define the alleviation mechanism.

Materials and methods

Plant material

Soybean (*Glycine max* L. Merrill) cv. Fukuyutaka was used as the plant material. Paddy soil mixed with 1 g compound fertilizer ($N:P_2O_5:K_2O = 3:10:10$) and 1 g lime were compacted in a plastic pod (0.7 L) before sowing. After emergence, one plant per pot was allowed, and the plants were grown at an air temperature of 25 °C and a relative humidity of 70% under natural daylength in a phytotron glass room in May 2009. Three weeks after emergence, either 1 mM H_2O_2 or distilled water (DW) was sprayed only once onto the leaves of each plant (100 mL/pot), and then irrigation was stopped. Treatments with H_2O_2 or DW were always followed by drought stress. The concentration of H_2O_2 was measured according to the method of López-Huertas et al. (2000). Measurements of all parameters were made after water had been withheld for 0, 2, 4, 6, and 8 days. For an additional control treatment, we included plants with no spray treatment and with irrigation maintained throughout the experiment. For RT-PCR and HPLC analysis, the leaves were collected at 0, 2, 4, 6, and 8 days after spraying and stored at −80 °C. The following measurements consisted of four replicates. The data were statistically evaluated by analysis of variance (ANOVA), and Tukey's test at $P < 0.05$ was used for the comparison of means.

Soil water content and leaf relative water content

At the completion of the treatment period, the soil down to a 3-cm depth was taken from each pot and weighed immediately to obtain its wet weight. The soil was then dried for 48 h at 90 °C, and the wet and dry weights were then used to estimate soil water content (SWC). SWC was calculated as [(soil wet weight) − (soil dry weight)]/(soil wet weight) × 100. To evaluate leaf relative water content (RWC), 50 leaf discs (5 mm in diameter) from each plant were weighed to determine fresh weight (FW), then hydrated to full turgidity by being floated in DW for 24 h at 4 °C and weighed again to determine the turgid fresh weight (TW). Dry weight (DW) was determined by drying for 48 h at 90 °C. RWC was then calculated as [(FW − DW)/(TW − DW)] × 100.

Photosynthetic measurements

Photosynthetic rate, stomatal conductance, and transpiration rate were measured in soybean leaves using an LCpro+ portable photosynthesis system (LCpro, ADC Bioscientific Ltd., UK) at room temperature (25 °C) in the morning (8:00–11:00am). The quantum flux density at the leaf surface, flow rate, and leaf temperature in the chamber were maintained at 1500 μmol m^{-2} s^{-1}, 200 μmol s^{-1}, and 30 °C, respectively. The rate of CO_2 assimilation in the chamber was measured at an ambient CO_2 concentration of 370 μL L^{-1}.

RT-PCR analysis

Total RNA was extracted from the soybean leaves using the SDS/phenol/LiCl method (Chirgwin et al., 1979), and cDNA was synthesized from total RNA (1 μg) using Rever TraACE reverse transcriptase (Toyobo, Japan) according to the manufacturer's protocol. cDNA (1 μL) was amplified in a reaction containing 10 μL of Go Taq Green Master Mix (Promega, USA), 0.1 μL each of 50 μM forward and reverse primers, and 8.8 μL of water. The amplification was conducted using a Program Temp Control System Astec PC-320 (Astec, Japan) as follows: 1 min at 94 °C; 27 cycles of 15 s at 94 °C, 30 s at 58–62 °C, and 30 s at 72 °C; then 5 min at 72 °C. The specific primer sequences for GmGolS (Glyma10g28610.1, Phytozome) were 5′-GACAAGCTTAAGCAGCAGATGGGGCACGGA-3′ and 5′-ATCGGATCCTGCCAGCAGCAGTGCCCCCATAAG-3′; for GmP5CS (Genbank accession no. AY492005), the specific primer sequences were 5′-ATCAAGAGTTCCACTAAAATTCCTGTC-3′ and 5′-TCATATGAGAAGGTCTCTGTGAGTGTAG-3′; for GmActin (Genbank accession no. V00450), the specific primer sequences were 5′-GCGTGATCTCACTGATGCCCTTAT-3′ and 5′-AGCCTTCGCAATCCACATCTGTTG-3′. The specific primer sequences for GmMIPSs were determined according to Chappell et al. (2006).

Myo-inositol and galactinol contents

Myo-inositol and galactinol levels were analyzed by HPLC using a 930-RI refractive index detector (JASCO, Japan) and a Shodex Asahipak NH2P-50 4E column (polymer-base, particle size; 5 μm, 250 mm × 4.6 mm i.d.) (Showa Denko K.K., Japan). Sugars were separated with acetonitrile–water (80:20, v/v) as an isocratic mobile phase at 0.8 mL/min using an 880PU pump (JASCO, Japan). The column was held at 40 °C.

Powdered freeze-dried leaves (50 mg) were extracted with 4 mL 80% (v/v) ethanol. The extracts were boiled for 20 min and centrifuged for 5 min at 25,000 × *g* to produce pellets of insoluble material. The supernatant was removed and the pellet was extracted twice more using the same approach. The supernatants were then combined and dried. The residue was dissolved in 1 mL of DW and passed through a Sep-Pak C18 mini-column (Waters, USA). The extracts were filtered (0.45 μm) before HPLC injection.

Results

Soil water contents and appearance of soybean plants during drought stress

The SWCs during the treatment period are shown in Fig. 1. The SWC in the control (unsprayed, irrigated) treatment was approximately 20% throughout the experiment. On the other hand, SWCs for the H_2O_2 and DW treatments significantly decreased, to approximately 8% at 4 days after treatment (DAT) and 4% at 8 DAT. The SWCs for the H_2O_2 and DW treatments were not significantly different from one another, suggesting that the level of drought in those treatments was the same.