Effect of Hydrogen Peroxide Spraying on Drought Stress in Soybean Plant

Yushi Ishibashi[a], Haruka yamaguchi[a], Takashi Yuasa[a], Mari Iwaya-Inoue[a], Susumu Arima[b,c], Shao-Hui Zheng[c]

[a] *Crop Science Lab., Faculty of Agricluture, Kyushu University, Hakozaki, Fukuoka 812-8581, Japan*
[b] *Faculty of Agriculture, Saga University, Saga 840-8502, Japan*
[c] *Coastal Bioenvironment Center, Saga University, Karatsu, Saga 840-0021, Japan*
** Corresponding author: Yushi Ishibashi, Crop Science Lab., Faculty of Agriculture, Kyushu University, Hakozaki, Fukuoka 812-8581, Japan. Tel.: +81 92 642 2824; fax: +81 92 642 2824.*
yushi@agr.kyushu-u.ac.jp

Abstract

We examined whether the spraying of soybean leaves with hydrogen peroxide (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 (*GmMIPS2*) 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.

Keywords: Drought stress, Galactinol, Hydrogen peroxide, Soybean

Introduction

Plants respond and adapt to water deficits at both the cellular and molecular levels by the accumulation of osmolytes and proteins that are specifically involved in stress tolerance. 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).

It is known that drought stress enhances the production of reactive oxygen species (ROS) in cellular compartments such as chloroplasts, peroxisomes, and mitochondria. If drought stress is prolonged, 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), controlling processes 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 *C. 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 materials

Soybean (*Glycine max* L. Merrill) cv. Fukuyutaka was used as the plant material. Three weeks after emergence, either 1 mM H_2O_2 or 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. 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. The following measurements consisted of four replicates.

Leaf relative water content

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 (8:00-11:00 am). 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 ºC, 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

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 to 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).

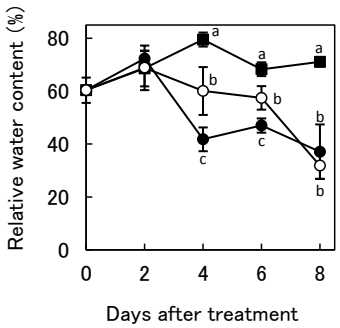
Myo-inositol and galactinol contents

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 and Discussion

The RWCs for the three treatments are shown in Figure 1. There were no significant differences in RWC between control, H_2O_2-treated, and DW-treated plants up to 2 DAT. At 4 DAT, the RWCs in control, H_2O_2-treated, and DW-treated plants were 80%, 60%, and 40%, respectively. The RWC at 6 DAT was also higher in H_2O_2-treated plants than in DW-treated plants. By 8 DAT, the RWCs for both the H_2O_2 and DW treatments were approximately 40%.



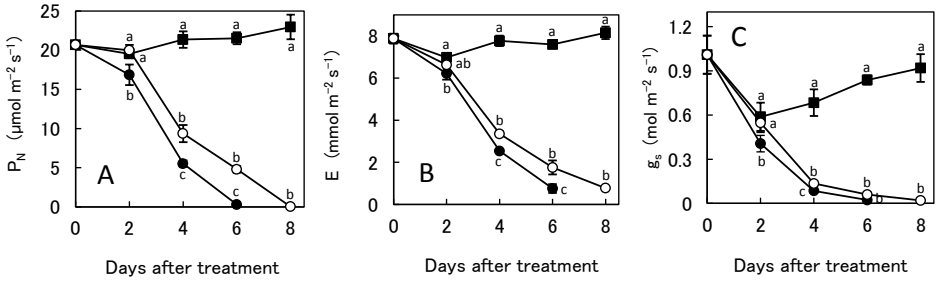
Closed squares indicate the control (irrigated, unsprayed) treatment. Open and closed circles indicate pre-treatment with H_2O_2 and DW respectively, followed by drought stress for the number of days indicated. For each day after treatment, means followed by different letters are significantly different at the 5% level as determined by Tukey's test (n = 4); error bars indicate standard deviations.

Figure 1. Relative water contents of soybean leaves during drought stress.

The net photosynthetic rate (P_N), transpiration rate (E), and stomatal conductance (g_s) during drought stress are shown in Figure 2. Here, although the P_N, E, and g_s significantly decreased during drought stress, these parameters were significantly higher in the H_2O_2-treated plants than in DW-treated plants. At 2 DAT, g_s levels in control, H_2O_2-treated, and DW-treated plants were 0.588, 0.508, and 0.323 mol m^{-2} s^{-1}, respectively (Fig. 2C). At 4 DAT, P_N levels in control, H_2O_2-treated, and DW-treated plants were 21.34, 9.36, and 5.51 µmol m^{-2} s^{-1}, respectively, and the levels of E were 7.76, 3.35, and 2.53 mmol m^{-2} s^{-1}, respectively (Fig. 2A, B).

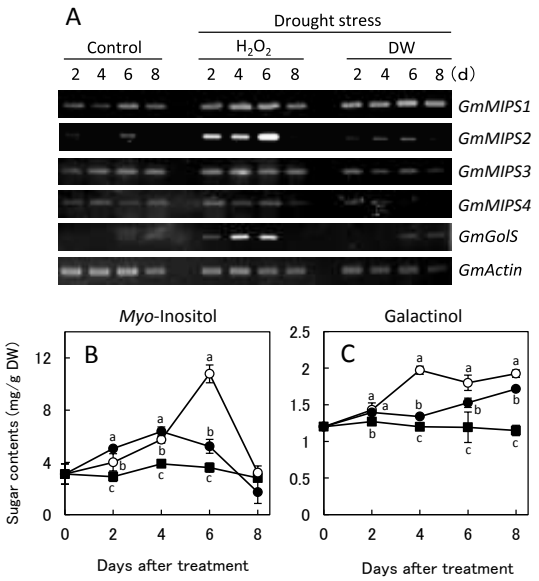
Myo-inositol plays critical and diverse biological roles in a myriad of cellular processes including signal transduction, stress responses, cell-wall biogenesis, growth regulation, and osmotolerance (Loewus and Murthy 2000). At 2, 4, and 6 DAT, *GmMIPS2* expression was markedly higher in leaves treated with H_2O_2 compared with control and DW-treated leaves (Fig. 3A). At 2 DAT, *GmMIPS2* expression was induced by H_2O_2 treatment but not by DW treatment. At 4 and 6 DAT, the expression of *GmMIPS2* mRNA in H_2O_2-treated leaves was remarkably higher than that in DW-treated leaves. In contrast, the expression of *GmMIPS1, -3,* and *-4* did not vary among the control, H_2O_2, and DW treatments. We also examined the expression of *galactinol synthase* (*GolS*),

which acts downstream of MIPS in the synthesis of raffinose-family oligosaccharides (RFOs). *GmGolS* expression was low in the control and DW-treated leaves but clearly higher in the H_2O_2–treated leaves, especially at 4 and 6 DAT (Fig. 3A).



(A) Photosynthetic rate (P_N); (B) transpiration rate (E); and (C) stomatal conductance (g_s). Closed squares indicate control (irrigated, unsprayed) treatment. Open and closed circles are H_2O_2 and DW pre-treatments, respectively, followed by drought stress for the number of days indicated. For each day after treatment, means followed by different letters are significantly different at the 5% level as determined by Tukey's test (n = 4); error bars indicate standard deviations.

Figure 2. Photosynthetic parameters during drought stress.



(A) Expression of *GmMIPS* genes and *GmGolS*. Control (irrigated, unsprayed); H_2O_2, H_2O_2 pre-treated; DW, DW pre-treated; d, days after treatment. *GmActin* was used as a control for loading. (B) *Myo*-inositol contents and (C) galactinol contents. Closed squares indicate the control (irrigated, unsprayed) treatment. Open and closed circles indicate pre-treatment with H_2O_2 and DW, respectively, followed by drought stress for the number of days indicated. Means followed by different letters are significantly different at the 5% level as determined by Tukey's test (n = 4); error bars

Figure 3. Gene expression of *GmMIPSs* and *GmGolS* and contents of *myo*-inositol and galactinol in leaves during drought stress.

We also found that the *myo*-inositol and galactinol contents in leaves pre-treated with H_2O_2 were increased compared with control and DW-treated leaves (Fig. 3B, C). *Myo*-inositol contents increased during drought stress, especially in H_2O_2-sprayed plants at 6 DAT, but decreased to starting levels by 8 DAT (Fig. 3B). The galactinol content in leaves pre-treated with H_2O_2 nearly doubled by 4 DAT, and the increased level was maintained until 8 DAT. On the other hand, the galactinol content in leaves pre-treated with DW increased until 8 DAT, but was significantly lower from 4 to 8 DAT than that seen in H_2O_2-treated plants (Fig. 3C).

Stress-inducible production of galactinol synthase (GolS) plays a key role in the accumulation of galactinol and raffinose, which function as osmoprotectants, under drought stress (Taji *et al*. 2002). GolS catalyzes the synthesis of galactinol from *myo*-inositol and UDP-galactose. We therefore examined the transcript levels of *D-myo-inositol 3-phosphate synthase1, -2, -3* and *-4* (*GmMIPSs*), which encode key enzymes in the synthesis of *myo*-inositol, and that of *GmGolS*, in H_2O_2-sprayed plants. We found that among the *GmMIPS* genes, only the transcript level of *GmMIPS2* increased after H_2O_2 spraying (Fig. 3A). Chappell *et al*. (2006) reported that *GmMIPS2* was poorly expressed in soybean leaves cultivated conventionally. The results reported here suggest that *GmMIPS2* is likely to be involved in drought stress signaling through ROS production caused by drought stress. Treatment with methylviologen, which enhances the production of O_2^- (another ROS), increased the transcript levels of *GolS* in *Arabidopsis thaliana* (Nishizawa *et al.* 2008). Overexpression of *AtGolS2* in transgenic *Arabidopsis thaliana* caused an increase in endogenous galactinol and raffinose, and showed improved drought tolerance (Taji *et al*. 2002). In soybeans, we have shown that H_2O_2 spraying increased the transcript level of *GmGolS* and the galactinol content in leaves (Fig. 3A, C). These results suggest that H_2O_2 spraying enabled the leaf to maintain a high level of RWC by regulating the osmolality in the leaf, consequently alleviating the effects of drought stress.

References

Azevedo Neto AD, Prisco JT, Eneas-Filho J, Medeiros JV, Gomes-Filho E. Hydrogen peroxide pre-treatment induces salt-stress acclimation in maize plants. 2005. *J. Plant Physiol.* 162: 1114-22.

Bailey-Serres J, Mittler R. 2006. The roles of reactive oxygen species in plant cells. *Plant Physiol.* 141: 311.

Boyer JS. 1982. Plant productivity and environment. *Science* 218: 443-8.

Bray EA, Bailey-Serres J, Weretilnyk E. 2000. Responses to abiotic stresses. In: Gruissem W, Buchannan B, Jones R, editors. Biochemistry and Molecular Biology of Plants. Rockville, MD. American Society of Plant Physiologists Pp1158-1249.

Chappell AS, Scaboo AM, Wu X, Nguyen H, Pantalone VR, Bilyeu KD. 2006. Characterization of the MIPS gene family in Glycine max. *Plant Breed.*125: 493-500.

Cruz de Carvalho MH. 2008. Drought stress and reactive oxygen species: production, scavenging and signaling. *Plant Signal Behav*. 3: 156-65.

Foyer CH, Lelandais M, Kunert KJ. 1994. Photooxidative stress in plants. *Physiol. Plant.* 92: 696-17.

Jubany-Marí T, Munné-Bosch S, López-Carbonell M, Alegre L. 2009. Hydrogen peroxide is involved in the acclimation of the Mediterranean shrub, *Cistus albidus* L., to summer drought. *J. Exp. Bot.* 60: 107-20.

Murata Y, Pei Z-M, Mori IC, Schroeder JI. 2001. Abscisic acid activation of plasma membrane Ca2+ channels in guard cells requires cytosolic NAD(P)H and is differentially disrupted upstream and downstream of reactive oxygen species production in abi1-1 and abi2-1 protein phosphatase 2C mutants. *Plant Cell* 13:2513-23.

Nishizawa A, Yabuta Y, Shigeoka S. 2008. Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. *Plant Physiol.*147: 1251-63.

Ozaki K, Uchida A., Takabe T, Shinagawa F, Tanaka Y, Takabe T, Hayashi T, Hattori T, Rai AK, Takabe T. 2009. Enrichment of sugar content in melon fruits by hydrogen peroxide treatment. *J Plant Physiol* .166: 569-78.

Pei Z-M, Murata Y, Benning G, Thomine S, Klusener B, Allen GJ, Grill E, Schroeder JI. 2000. Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature* 406: 731-4.

Schroeder JI, Allen GJ, Hugouvieux V, Kwak JM, Waner D. 2001. Guard cell signal transduction. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 52: 627-58.

Taji T, Ohsumi C, Iuchi S, Seki M, Kasuga M, Kobayashi M, Yamaguchi-Shinozaki K, Shinozaki K. 2002. Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. *Plant J*. 29: 417-26.

Wang P, Song C. 2008. Guard-cell signalling for hydrogen peroxide and abscisic acid. *New Phytol*.178: 703-18.

-- back to Table of Content --