Effects of Exogenous Spermine on Polyethylene Glycol-induced Responses in Rice Leaves

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ABSTRACT

Polyamines are low-molecular weight polycations involved in many physiological processes in plants. In this work, we studied the effects of spermine (SPM) on the responses in detached rice (Oryza sativa L.) leaves caused by water stress. Polyethylene glycol 6000 (PEG) was used to induce water stress of detached rice leaves. Upon treatment with PEG, the contents of H$_2$O$_2$, malondialdehyde, NH$_4^+$, abscisic acid (ABA), and proline increased in detached rice leaves. PEG treatment also resulted in chlorophyll loss and protein degradation in detached. ABA content in detached rice leaves was increased by exogenous application of H$_2$O$_2$. SPM pretreatment was effective in reducing PEG-induced H$_2$O$_2$ production, lipid peroxidation, NH$_4^+$ accumulation, chlorophyll loss, protein degradation, and ABA elevation in detached. However, SPM pretreatment had no effect on PEG-induced reduction of relative water content and accumulation of proline. It appears that SPM does not protect against all the responses of detached rice leaves induced by PEG.

Key words: Hydrogen peroxide, Oryza sativa L., Spermine, Water stress.

INTRODUCTION

Inadequate water availability is a crucial limitation to crop growth and yield (Boyer 1982, Saini and Westgate 2000, Sharp et al. 2000). During water stress, leaf stomatal closure limits water loss and the influx of CO$_2$. Lowered CO$_2$ influx leads to a decrease in carbon reduction by the Calvin cycle and to a decrease in oxidized NADP$^+$ to serve as an electron acceptor in photosynthesis. As a result, electrons flow to the alternative electron acceptor, O$_2$ and produce superoxide radical and consequently other reactive oxygen species (ROS) including H$_2$O$_2$ (Scandalios 1993, Smirnoff 1993, Miller et al. 2008).

Numerous studies indicate that H$_2$O$_2$ content and lipid peroxidation increase during water stress (Chowdhury and Choudhuri 1985, Kubis 2008).
Sairam et al. 1998, Yong and Jung 1999, Luna et al. 2005, Selote and Khanna-Chopra 2006). \( \text{H}_2\text{O}_2 \) plays a dual role acting on the one hand as a toxic compound and on the other as an important signal transduction molecule during stress conditions (Crux de Carvalho 2008, Miller et al. 2008).

Polyamines, which are low-molecular weight polycations, are found in living cells. The common polyamines in plants are spermidine (SPD), spermine (SPM) and their precursor, putrescine (PUT). They are implicated in many physiological processes such as growth, morphogenesis, secondary metabolism, senescence, and apoptosis (Bouchereau et al. 1999, Kuehn and Phillips 2005, Kusano et al. 2008). In recent years, attention has been focused on the role of polyamines in plants defence against abiotic and biotic stresses (Galston 2001, Ma et al. 2005, Liu et al. 2006, Kusano et al. 2007, 2008, Yang et al. 2007).

The protective effect of polyamines against PQ toxicity has been described in plant systems (Chang and Kao 1997, Kurepa et al. 1998, Benavides et al. 2000). Polyamins have also been reported as direct free radical scavengers (Drolet et al. 1986). Evidence has been provided to show that the reduction in polyamine contents in leaves of *Glycyrrhiza inflata* under osmotic stress (Li and Wang 2004). It has been shown that oxidative damage caused by ozone and Cd is reduced by exogenous application of polyamins (Ormrod and Beckerson 1986). Hsu and Kao 2007). However, Bors et al. (1989) claimed that the scavenging of radicals by polyamines cannot explain the protection against ozone damage after exogenous application. Recently, Yamaguchi et al. (2006) found that a SPM-deficient Arabidopsis mutant exhibits hypersensitivity to NaCl stress. NaCl-hypersensitivity of the mutant can be cured by SPM but not by PUT and SPD, suggesting a close-link between SPM-deficiency and NaCl-hypersensitivity.

Rice is a paddy field crop and is particularly susceptible to water deficit. However, the mechanisms of drought tolerance in rice plants remain poorly understood. Recent experiments by Yang et al. (2007) suggested that in adapting to water deficit it would be good for rice to have the physiological traits of higher levels of SPD/SPM under water stress.

Previously, we have shown that PEG induced the increase in malondialdehyde (MDA) content, routinely used as an indicator of lipid peroxidation, the accumulations of \( \text{NH}_4^+ \), proline, and abscisic acid (ABA), and the decrease in chlorophyll and protein contents in rice leaves (Cheng et al. 2002, Hsu and Kao 2003, Hsu et al. 2003a, b). Here we examined whether SPM protected against all these effects induced by PEG. Our results indicated that SPM does not protect against all the responses in rice leaves induced by PEG.

**MATERIALS AND METHODS**

**Plant material**

Rice (*Oryza sativa* L. cv. Taichung Native 1) seeds were sterilized with 2.5% sodium hypchlorite for 15 min and washed extensively seeds with distilled water. These seeds were then germinated in Petri dishes with wetted filter paper at 37°C under dark conditions. After 48 h incubation, uniformly germinated seeds were selected and cultivated in a 500 mL beaker containing half-strength Kimura B solution as described previously (Hsu and Kao 2005). The hydroponically cultivated seedlings were grown for 12 d in a Phytotron (Agricultural Experimental Station, National Taiwan University, Taipei, Taiwan) with natural sunlight at 30°C day/25°C night and 90% relative humidity. The apical 3 cm of the third leaf was used for all experiments. A group of 10 segments floated in a Petri dish containing 10 mL of distilled water were served as controls. For induction of water stress, leaf segments were exposed to PEG solution of osmotic potential -1.5 MPa. All sample were kept at temperature at 27°C and irradiance of 40 µmol m\(^{-2}\)s\(^{-1}\). To examine the effects of SPM pretreatment on the effects caused by PEG, detached rice leaves were pretreated with either water or 5 mM SPM for 6 h in the dark and then transferred to water or PEG for 12 h in the light.

**Estimation of water deficit**

Water deficit of leaves was estimated by leaf rolling (O’Toole and Cruz 1990) or relative water content (RWC). RWC, defined as water content of leaf tissue as a percentage that of the fully turgid tissue, was determined by the method of Weatherley (1950).
Determination of $H_2O_2$, MDA, chlorophyll, protein, $NH_4^+$, proline, and ABA

The $H_2O_2$ content was measured colorimetrically as described by Jana and Choudhuri (1982). $H_2O_2$ was extracted by homogenizing leaf tissue with phosphate buffer (50 mM, pH 6.5) containing 1 mM hydroxylamine. The homogenate was centrifuged at 6,000 x $g$ for 25 min. To determine $H_2O_2$ content, the extracted solution was mixed with 0.1% titanium chloride in 20% (v/v) $H_2SO_4$. The mixture was then centrifuged at 6,000 x $g$ for 15 min. The absorbance was measured at 410 nm. The $H_2O_2$ content in leaf extracts was calculated using the extinction coefficient of 0.28 µmol⁻¹ cm⁻¹.

MDA was extracted with 5% (w/v) trichloroacetic acid and determined by the thiobarbituric acid reaction as described by Heath and Packer (1968). Chlorophyll content was determined according to Wintermans and De Mots (1965) after extraction in 96% ethanol. Protein was determined by the method of Bradford (1978).

$NH_4^+$ was extracted by homogenizing leaf segments with a pestle and mortar using 0.3 mM sulfuric acid (pH 3.5). The homogenate was centrifuged for 10 min at 39,000 x $g$. The supernatant was used to determine $NH_4^+$ content by the method of Weatherburn (1967). $NH_4^+$ content was calculated using an extinction coefficient of 3.91 µmol⁻¹ cm⁻¹.

Proline was extracted and its concentration determined by the method of Bates et al. (1973). Leaf segments were homogenized with 3% sulfosalicylic acid and the homogenate was centrifuged at 3,000 x $g$ for 20 min. The supernatant was treated with acetic acid and acid ninhydrin, boiled for 1 h, and then absorbance at 520 nm was determined.

For extraction of ABA, leaves were homogenized with a pestle and mortar in extraction solution (80% methanol containing 2% glacial acetic acid). To remove plant pigments and other non-polar compounds which could interfere in the immunoassay, extracts were first passed through polyvinylpyrrolidone column and C18 (Sep-Pak Vac) cartridges (Waters, Milford, MA). The eluates were concentrated to dryness by vacuum-evaporation and resuspended in Tris-buffered saline before enzyme-linked immunosorbent assay (ELISA). ABA was quantified by ELISA (Walker-Simmons 1987). ABA immunoassay detection kit (PGR-1) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) is specific for (+)-ABA. By evaluating $^3$H-ABA recovery, $^3$H-ABA loss was less than 3% by the method described here.

Statistical analysis

The contents of $H_2O_2$, MDA, $NH_4^+$, proline, chlorophyll, protein, and ABA were expressed on the basis of initial fresh weight (FW). Statistical differences between measurements ($n = 4$) on different treatments or on different times were analyzed following Student’s $t$-test or LSD test.

RESULTS

Time course of the changes caused by PEG treatment

Detached rice leaves were treated with water and PEG for various times in the light. Leaf rolling of rice leaves treated with PEG was clearly observed at 0.5 h after treatment (Fig. 1A). The increase in $H_2O_2$ was evident 2 h after treatment (Fig. 1B). MDA content remained almost no changes in control leaves during 4-h incubation (Fig. 1C). However, there was a significant increase in MDA content in PEG-treated leaves 4 h after treatment (Fig. 1C). Chlorophyll and protein contents decreased 3 h after PEG treatment (Figs. 2A and B). PEG-treated rice leaves had higher $NH_4^+$ content than the controls at 4 h after treatment (Fig. 3A). Higher contents of proline and ABA were observed at 1 and 3 h after PEG treatment, respectively (Figs. 3B and C).

SPM pretreatment

The effects of SPM pretreatment on PEG-induced responses are shown in Figs. 4 and 6. SPM pretreatment was observed to be effective in reducing PEG-induced $H_2O_2$ production (Fig. 4A), lipid peroxidation (Fig. 4B), chlorophyll loss (Fig. 4C), protein degradation (Fig. 4D), $NH_4^+$ accumulation (Fig. 4E) and ABA elevation (Fig. 4F). However, SPM pretreatment had no effect on RWC reduction (Fig. 6A) and proline accumulation (Fig. 6B).

Effect of exogenous $H_2O_2$ on ABA content

To study whether exogenous $H_2O_2$ increases ABA content, detached rice leaves were treated
with 10 mM H$_2$O$_2$ in the light. Fig. 5 shows that H$_2$O$_2$ treatment resulted in an increase in ABA content in rice leaves.

**DISCUSSION**

In the present study, we show that PEG treatment induced H$_2$O$_2$ production in rice leaves (Fig. 1B). The production of H$_2$O$_2$ by water stress has also been described previously (Chowdhury and Choudhuri 1985, Sairam et al. 1998, Yong and June 1999, Luna et al. 2005, Selote and Khanna-Chopra 2006, Kubis 2008). Wounding is known to induce H$_2$O$_2$ production (Orozco-Cárdenas and Ryan 1999). When detached leaves were used to study H$_2$O$_2$

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**Fig. 1.** Changes in leaf rolling (A) and the contents of H$_2$O$_2$ (B) and MDA (C) in rice leaves treated with PEG (-1.5 MPa). Detached rice leaves were treated with H$_2$O or PEG in the light. Bars indicate standard error ($n = 4$). * represents values that are significantly different between H$_2$O and PEG treatments at $P < 0.05$ by using Student’s $t$-test.

**Fig. 2.** Changes in the contents of chlorophyll (A) and protein (B) in rice leaves treated with PEG (-1.5 MPa). Detached rice leaves were treated with H$_2$O or PEG in the light. Bars indicate standard error ($n = 4$). * represents values that are significantly different between H$_2$O and PEG treatments at $P < 0.05$ by using Student’s $t$-test.

**Fig. 3.** Changes in the contents of NH$_4^+$ (A), proline (B), and ABA (C) in rice leaves treated with PEG (-1.5 MPa). Detached rice leaves were treated with H$_2$O or PEG in the light. Bars indicate standard error ($n = 4$). * represents values that are significantly different between H$_2$O and PEG treatments at $P < 0.05$ by using Student’s $t$-test.
Fig. 4. Effect of SPM pretreatment on the contents of H$_2$O$_2$ (A), MDA (B), chlorophyll (C), protein (D), NH$_4^+$ (E), and ABA (F) in detached rice leaves in the presence or absence of PEG (-1.5 MPa). Detached rice leaves were pretreated with H$_2$O or 5 mM SPM for 6 h in the dark and then treated with H$_2$O or PEG for 12 h in the light. Bars indicate standard error (n = 4). Values with the same letter are not significantly different at P < 0.05 by using LSD test.

Fig. 5. Effect of H$_2$O$_2$ on ABA content in rice leaves. Detached rice leaves were treated with H$_2$O and 10 mM H$_2$O$_2$ for 12 h in the light. Bars indicate standard error (n = 4). * represents values that are significantly different between H$_2$O and H$_2$O$_2$ treatments at P < 0.05 by using Student’s t-test.

production, wounding is always a problem. However, in the present study, each long and narrow rice leaf was cut transversally, thus, the area of wounding was very small. Therefore, H$_2$O$_2$ production of detached rice leaves induced by PEG (Fig. 1B) is unlikely to be complicated by the wounding effect.

As a stress hormone, ABA plays an important role under water stress and it may accumulate in response to water stress (Zeevaart and Creelman 1988). Here, we also observed that PEG treatment resulted in an increase in ABA content in rice leaves (Fig. 3C). Hu et al. (2005, 2006) reported that ABA is a key inducer of H$_2$O$_2$ production in leaves of maize plans exposed to water stress. Here, we show that PEG-induced H$_2$O$_2$ production occurred before PEG-induced ABA accumulation in rice leaves (Figs. 1B and 3C). Thus, H$_2$O$_2$ production in rice leaves caused by
Fig. 6. Effect of SPM pretreatment on RWC (A) and proline content (B) in detached rice leaves in the presence or absence of PEG (-1.5 MPa). Detached rice leaves were pretreated with H$_2$O or 5 mM SPM for 6 h in the dark and then treated with H$_2$O or PEG for 12 h in the light. Bars indicate standard error (n = 4). Values with the same letter are not significantly different at P < 0.05 by using LSD test.

PEG is unlikely to be mediated through ABA. In plants, H$_2$O$_2$ can be generated by a cell wall-localized peroxidase, amine oxidases, oxalate oxidase and NADPH oxidase (Dumas et al. 1995, Bolwell et al. 1998, Papadakis and Roubelakis-Angelakis 1999, Yoda et al. 2003, Pourrut et al. 1998). Work is underway to determine the possible enzymes responsible for H$_2$O$_2$ generation in PEG-treated rice leaves.

Over-production of ROS in chloroplasts of plants under drought stress has been suggested to be the major factor responsible for oxidative damage in leaves (Foyer and Noctor 2003). Thus, the production of H$_2$O$_2$ caused by PEG treatment may lead to the oxidation of macromolecules, which can induce lipid peroxidation. In the present study, exogenous application of SPM significantly decreased H$_2$O$_2$ and MDA contents under PEG stress (Figs. 4A and B). In addition, it was found that detached rice leaves pretreated with SPM for 6 h in the dark had higher endogenous level of SPM than those pretreated with water (Hsu and Kao 2007). Thus, our results may suggest that SPM can alleviate the lipid peroxidation via decreasing H$_2$O$_2$ production in rice leaves.

Glutamine synthetase (GS), the key enzyme in the generally recognized GS/glutamate synthase pathway, plays a crucial role in the assimilation of NH$_4^+$ (Miflin and Lea 1976). Here, we show that NH$_4^+$ content in detached rice leaves exposed to PEG was greater than that in control leaves (Fig. 3A) and that SPM significantly decreased NH$_4^+$ content in PEG-treated rice leaves (Fig. 4E). It has been shown that PEG-induced NH$_4^+$ accumulation is attributed to the decrease in GS activity (Hsu et al. 2003b). GS in plants has been reported to be particularly prone to degradation under oxidative stress conditions (Ortega et al. 1999, Palatnik et al. 1999, Chien et al. 2002, Ishida et al. 2002). It is most likely that the alleviation of PEG-induced NH$_4^+$ accumulation by SPM is mediated through decreasing H$_2$O$_2$ content.

Since H$_2$O$_2$ production increased before ABA accumulation in detached rice leaves exposed to PEG (Figs. 1B and 3C), it is possible that H$_2$O$_2$ is involved in PEG-induced ABA accumulation. If this hypothesis is correct, then SPM treatment, which reduced H$_2$O$_2$ production in PEG-treated rice leaves, is able to alleviate the accumulation of ABA. As indicated in Figs. 4A and F, it should be the case. In agreement with these results, exogenous application of H$_2$O$_2$ can also induce ABA accumulation in rice leaves (Fig. 5). The involvement of ROS in drought-induced ABA accumulation has also been described in root tips of wheat seedlings (Zhao et al. 2001).

The accumulation of osmolytes during stress is well documented. There have been reports that proline accumulation in plants under water stress (Yoshiba et al. 1997, Hsu et al. 2003a). Previously, we demonstrated that NH$_4$Cl and methionine sulfoximine (an inhibitor of GS) treatments, which caused NH$_4^+$ accumulation, increase proline content in detached rice leaves (Yang and Kao 1999). However, proline accumulation observed in PEG-treated rice leaves was not a result of NH$_4^+$ accumulation, because proline
accumulation occurred before NH$_4^+$ accumulation (Figs. 3A and B). It has been shown that ABA accumulation is required for proline accumulation in maize primary roots at lower water potentials (Ober and Sharp 1994). However, proline accumulation in the absence of ABA accumulation has also been reported (Stewart and Voetberg 1987, Yoshiba et al. 1997). The time-course analyses of changes in rice leaves caused by PEG clearly indicate that proline accumulation occurs first and then ABA increases (Figs. 3B and C). Thus, proline accumulation in PEG-treated rice leaves is not due to ABA accumulation (Fig. 6). The failure of SPM in reducing PEG-induced RWC reduction and proline accumulation in rice leaves can be explained by the observations that H$_2$O$_2$ content increased after proline accumulation (Figs. 1B and 3B). It appears that PEG-induced proline accumulation is directly through water loss rather than H$_2$O$_2$ accumulation.

Results obtained in the present study suggest that in detached rice leaves there are two types of PEG effects: H$_2$O$_2$-independent and H$_2$O$_2$-dependent. Our data also show that SPM is able to reduce H$_2$O$_2$ content increased by PEG (Fig. 4A) and that only PEG-induced lipid peroxidation, chlorophyll loss, protein degradation, NH$_4^+$ accumulation and ABA elevation, which occurred after H$_2$O$_2$ accumulation, can be reduced by SPM pretreatment (Figs. 4B-F). It appears that accumulation of H$_2$O$_2$ in PEG-treated rice leaves signals the increase in lipid peroxidation, NH$_4^+$ accumulation, chlorophyll loss, protein loss, and ABA elevation (Fig. 7). The inability of SPM in reducing water loss (Fig. 5A) suggests that osmotic adjustment in detached rice leaves cannot be regulated by SPM under drought conditions.

Regulation of PEG-induced responses by SPM in detached rice leaves as we observed here is not necessarily similar to that in intact leaves under field conditions. However, the results of the present work do provide some basic information which should be valuable for our future studies.

Fig. 7. Proposed regulation of PEG-induced changes in rice leaves by SPM.
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