Tree Physiology 30, 914–922
doi:10.1093/treephys/tpq030

Spermine pretreatment confers dehydration tolerance of citrus in vitro plants via modulation of antioxidative capacity and stomatal response

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Received November 24, 2009; accepted March 16, 2010; published online May 12, 2010

Summary Polyamines, small aliphatic polycations, have been suggested to play key roles in a number of biological processes. In this paper, attempts were made to investigate the possibility of improving dehydration tolerance of citrus in vitro plants by exogenous application of spermine (Spm). 'Red Tangerine' (*Citrus reticulata* Blanco) in vitro plants pretreated with 1 mM Spm exhibited less wilted phenotype and lower water loss and electrolyte leakage than the control under dehydration. Spm-pretreated plants contained higher endogenous polyamine content during the course of the experiment relative to the control, particularly at the end of dehydration, coupled with higher expression levels of *ADC* and *SPMS*. Histochemical staining showed that the Spm-pretreated leaves were stained to a lower extent than those without Spm pretreatment, implying generation of less reactive oxygen species (ROS). On the contrary, activities of peroxidase (POD) and superoxide dismutase (SOD) in the Spm-pretreated samples were higher than the control at a given time point or during the whole experiment, suggesting that Spm exerted a positive effect on antioxidant systems. In addition, significantly smaller stomatal aperture size was observed in Spm-pretreated epidermal peels, which showed that stomatal closure was promoted by polyamines. All of these data suggest that Spm pretreatment causes accumulation of higher endogenous polyamines and accordingly leads to more effective ROS scavenging (less tissue damage) and stimulated stomatal closure (lower water loss) upon dehydration, which may function collectively to enhance dehydration tolerance.

Keywords: antioxidant enzymes, Citrus reticulata, dehydration tolerance, polyamine, reactive oxygen species, stomatal response.

Introduction

The citrus industry worldwide is always threatened by adverse environmental stresses, among which drought is one of the most devastating factors. Drought retards plant growth, reduces fruit size and yield and promotes leaf abscission, leading to irreversible damage in some cases (García-Sánchez et al. 2007). Accordingly, it is important to develop appropriate strategies that can be taken to tackle the drought stress. Although selection and breeding of drought-tolerant cultivars have been suggested to be an effective solution to this issue, progress in drought-tolerance-oriented breeding has been fairly slow in *Citrus* due to several reproductive barriers, such as polyembryony, long juvenility, high heterozygosity and male/female sterility. Therefore, it is still a favorable way to take other measures in order to minimize drought-derived stress damage.

Drought results in water deficit and loss of cell turgor. In addition, it evokes overproduction of highly reactive oxygen species (ROS) like superoxide (O_2^-) and hydrogen peroxide (H_2O_2), leading to oxidative stress. It has been well documented that plants have developed an array of mechanisms to cope with these abnormal physiological disorders. Accumulating evidence has been acquired to show that under stressful conditions plants may undergo physiological, biochemical, cellular and molecular alterations (Yamaguchi-Shinozaki and Shinozaki 2005). One approach for the plants to respond and adapt to adverse milieus is the accumulation of compatible solutes, also known as osmoprotectants, for osmotic adjustment and maintenance of cell turgor. Moreover, the plants evolve an endogenous defensive mechanism to protect cellular and subcellular components against oxidative stress, in which ROS is primarily removed by enzymatic or non-enzymatic antioxidant systems (Arbona and Gómez-Cadenas 2008). These two mechanisms might work independently or in synergy to mitigate stress-induced cell death and consequently enhance stress tolerance. It is thus conceivable that a given compound that can function both as osmoprotectant and ROS scavenger will serve as a robust effector to counteract the drought stress. In this regard, polyamines can be regarded as a satisfactory candidate to meet the two requirements.

Polyamines, primarily spermidine (Spd), spermine (Spm) and their diamine precursor, putrescine (Put), are low-

molecular-weight aliphatic polycations that are ubiquitously present in almost all living organisms. Being positively charged at physiological pH, they can interact with various cellular macromolecules like nucleic acids, protein and membrane phospholipids and regulate relevant processes (Martin-Tanguy 2001). These properties provide the basis for the involvement of polyamines in a wide spectrum of physiological processes, including cell division, embryogenesis, morphogenesis, growth and development (Evans and Malmberg 1989, Liu et al. 2006*a*). Moreover, intensive work has revealed that polyamines play important roles in stress response, although the definitive modes of action remain a matter of speculation (Liu et al. 2007, Kusano et al. 2008). So far, a growing body of research has revealed accumulation of polyamines in many plants upon exposure to a variety of stresses, including salinity, chilling, drought, ozone and pathogen invasion (Liu et al. 2007, Kusano et al. 2008, references therein). Based on these phenomena, it has been proposed to enhance stress tolerance through augmenting endogenous cellular polyamine content via either genetic transformation or exogenous replenishment of polyamines. For example, transformation of an *arginine decarboxylase* gene *Datura stramonium* in rice led to remarkable drought tolerance (Capell et al. 2004). Interestingly, over-expression of *Cucurbita ficifolia spermidine synthase* (*SPDS*) gene in *Arabidopsis thaliana* and apple *SPDS* gene (*MdSPDS1*) in European pear confers tolerance to multiple abiotic stresses (Kasukabe et al. 2004, Wen et al. 2008, 2009). Exogenously applied polyamines have also been shown to effectively alleviate stress-derived injury caused by acid rain (Velikova et al. 2000), ozone (Navakoudis et al. 2003), heavy metals (Zhao and Yang 2008), chilling (Shen et al. 2000), salinity (Iqbal and Ashraf 2005, Liu et al. 2006*b*) and water stress (Kubiś 2008, Farooq et al. 2009, Yiu et al. 2009).

The above-mentioned illustration suggests that modulation of cellular polyamine content can be regarded as a convenient and effective strategy to enhance stress tolerance. However, it is noted that most previous studies were carried out on annual plants, whereas little information is available about the role of exogenous polyamines in combating water stress imposed on perennial plants. To address this issue, in the current work attempts were made to investigate whether pretreatment of polyamines prior to short-term severe drought (dehydration at ambient environment) can render stress tolerance of citrus in vitro plants. One purpose of this study is to provide clues for application of polyamines in citrus production during the dry season. The other purpose of this work is to offer new evidence supporting polyamines' protective roles against oxidative stress caused by water deficit.

Materials and methods

Plant materials and treatments

For establishment of in vitro plants, seeds of 'Red Tangerine' (*Citrus reticulata* Blanco) were surface sterilized and sown on germination medium (GM) containing salts of MT (Murashige and Tucker 1969) medium, 30 g l^{-1} sucrose and 7 g l^{-1} agar (pH 5.8). The materials were kept under a 16-h light (provided by incandescent light, at approximately 75 μmol m^{-2} s^{-1})/8-h dark regime in a culture room at 25 ± 1 °C. 'Red Tangerine' in vitro plantlets were incubated for 5 days in liquid 1/2GM supplemented with 1 mM of Spm (Arasimowicz-Jelonek et al. 2009), whereas those incubated only in liquid 1/2GM were considered as control. Both the Spm-treated and the control plants were removed from the solution, gently washed with distilled water and quickly filter dried to eliminate the liquid solution on the root surface and free spaces. The plantlets were then put on Petri dishes at ambient environment in the daytime for up to 12 h. Fresh weight and electrolyte leakage (EL) of the plantlets were measured 0, 1, 3 and 12 h after dehydration (initiation of the dehydration stress was considered as time point '0'). Samples were collected at the same time, which were immediately frozen in liquid nitrogen and stored at −80 °C until use, except those for scanning electron microscopy assay, which were harvested at 0 and 12 h and fixed immediately after sampling.

Measurement of EL

Measurement of EL was performed based on a method described by Liu et al. (2006*b*) with slight modification. Leaves from the samples collected at each time point were stripped and incubated in 10 ml distilled water and shaken on a gyratory shaker (200 rpm) at room temperature for 1 h, and the initial conductivity (C1) was measured with a conductivity meter (DSS-307; Shanghai, China). The samples were then boiled for 10 min to induce complete leakage and cooled down to room temperature before measurement of electrolyte conductivity (C2). The electrolyte leakage (*C*) was calculated according to the equation C (%) = 100 × C1/C2.

In situ histochemical localization of O_2^- and H_2O_2

In situ accumulation of O_2^- and H_2O_2 was detected by histochemical staining with nitro blue tetrazolium (NBT) and diaminobenzidine (DAB) according to Romero-Puertas et al. (2004) with minor modification. For O_2^- detection, the leaves of control and Spm-pretreated plantlets under dehydration treatment were excised and immersed in a 1 mg ml^{-1} solution of NBT in 10 mM phosphate buffer (pH 7.8) at room temperature. The immersed leaves were illuminated for 1–2 h until appearance of dark spots, characteristic of blue formazan precipitates. For localization of H_2O_2, another set of leaves was sampled and immersed in DAB solution (1 mg ml^{-1}, pH 3.8) that was freshly made in 10 mM phosphate buffer (pH 7.8), and incubated at room temperature for 8 h in the light until brown spots were visible, which are derived from the reaction of DAB with H_2O_2. For both staining methods, the leaves were then bleached in concentrated ethanol to visualize the blue and brown spots, which were kept in 70% ethanol for taking pictures by a digital camera.

Extraction and analysis of superoxide dismutase and guaiacol-peroxidase

For extraction of superoxide dismutase (SOD) (EC 1.15.1.1) and guaiacol-peroxidase (POD) (EC 1.11.1.7), 0.2 g of leaf sample was ground using a cold mortar and pestle in liquid nitrogen and then homogenized in 3.0 ml of extraction buffer composed of 50 mM phosphate buffer (pH 7.8) and 1% polyvinylpyrrolidone, followed by centrifugation for 20 min at 10 000 rpm. Unless otherwise stated, all operations were carried out at 4 °C until the enzyme determination. The resulting supernatant was used for enzyme analysis at 25 °C by spectrophotometry. SOD activity was determined according to the method described by Huang et al. (2008) with some modification. Three milliliters of reaction mixture consisted of 50 mM phosphate buffer (pH 7.0), 130 mM methionine, 0.75 mM NBT (an indicator of superoxide radical production) and 0.1 mM EDTA-Na_2. One unit of SOD was defined as 50% inhibition of NBT under assay conditions. POD activity was assayed according to Chen and Wang (2002). The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 0.2% guaiacol, 0.3% H_2O_2 and enzyme extract. Activity of POD was determined by the increase in absorbance at 470 nm due to guaiacol oxidation, and 1 unit of the enzyme activity was defined as an increase of 0.01 per min in the absorbance.

Quantification of free polyamines

Free polyamines were extracted and measured according to He et al. (2002) and Wu et al. (2009) with minor modifications. In brief, about 0.1 g of sample powder was homogenized in 1 ml of cold 5% perchloric acid (PCA) containing 500 mg l^{-1} 1,4 dithiothreitol (DTT) and kept on ice for 30 min. After 10 min of centrifugation at 15 000 rpm (4 °C), the supernatant fraction was collected and kept on ice. The resulting pellet was extracted in 1 ml of PCA and kept for 30 min on ice before centrifugation with the same conditions as mentioned above. The supernatant from two rounds of centrifugation was combined, from which 200 µl was transferred to a 0.5-ml tube containing 200 µl saturated sodium bicarbonate, 5 µl 1,6-hexanediamine (100 µM stock, used as an internal standard) and 400 µl dansyl chloride (10 mg ml^{-1} in acetone). After 60 min incubation at 60 °C, 100 µl proline (100 mg ml^{-1}) was added, and the mixture was incubated for an additional 30 min at 60 °C. Thereafter, 400 µl of toluene was added and thoroughly mixed, followed by centrifugation at 10 000 rpm for 5 min. Then 400 µl of the toluene phase (upper one) was transferred to a new tube and dried under vacuum, to which 1 ml of high performance liquid chromatography (HPLC)-grade methanol was added for suspending the dansylated polyamine. Twenty microliters of the prepared polyamine solutions was loaded to a HPLC (Waters, USA) equipped with a C_{18} reversed-phase column (4.6 mm × 150 mm, particle size 5 µm) with a programmed gradient of solvents (methanol/water), changing from 60 to 95% in 23 min at a flow rate of 1 ml min^{-1}. The eluates were detected via a fluorescence spectrophotometer with excitation and emission wavelength of 365 and 510 nm, respectively. Quantification of free polyamines was done in triplicate for each treatment.

Analysis of expression level of polyamine biosynthetic genes by reverse transcriptase polymerase chain reaction

Total RNA of the samples collected at 0, 1 and 12 h after dehydration was extracted using TRIZOL according to Liu et al. (2006), and cDNAs were synthesized from 1 µg of total RNA in a 20-µl reaction volume with a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Lithuania) following the manufacturer's instructions. Gene expression profiles of *ADC* (arginine decarboxylase), *ODC* (ornithine decarboxylase), *SAMDC* (*S*-adenosylmethionine decarboxylase), *SPDS*, *SPMS* (spermine synthase) and actin (used as internal control) were assayed via reverse transcriptase polymerase chain reaction (RT–PCR). PCR reaction cocktail consisted of 100 ng of cDNA, 1× reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 U *Taq* polymerase and 0.2 µM forward primer and 0.2 µM reverse primer (Wu et al. 2009) in a total volume of 20 µl. The PCR program consisted of 5 min incubation at 94 °C followed by 28 cycles of 40 s at 94 °C, 40 s at 52 °C and 40 s at 72 °C, and 1 cycle of 6-min extension at 72 °C. After the amplification, the RT–PCR production was separated in 1.25% agarose gel containing 0.5 µg ml^{-1} ethidium bromide and visualized under UV transillumination.

Stomatal aperture measurement

Stomatal aperture at 0 and 12 h after dehydration in the Spm-treated and control plants was examined by scanning electron microscopy (SEM). Ultrathin leaf sections were made as previously described (Dong et al. 2009) and observed by a scanning electronic microscope (H-7650; Hitachi, Ltd., Tokyo, Japan), and the images were collected as JPG digital files. Width of aperture of at least 70 stomata was measured using ImageJ software (version 1.4.3.67) for each time point.

Statistical analysis

Pretreatment of polyamines and dehydration were repeated three times, producing similar trends. Therefore, all of the presented data are mean values of a representative experiment (four plants) and shown as the mean ± SE. The data were analyzed using SAS statistical software, and analysis of variance (ANOVA) was used to compare the statistical difference based on Duncan's multiple range test, at significance level of $P < 0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***).

Results

Water loss and electrolyte leakage of Spm-treated and control 'Red Tangerine' plants under dehydration

'Red Tangerine' plants without Spm pretreatment (control) showed progressive water loss (Figure 1A), 18.9, 33.7 and 52.5% at 1, 3 and 12 h after the dehydration, respectively. Compared with the control, slower and less water loss was observed in the Spm-treated plants, as shown by 10.4, 14.7 and 29.1% at the same time points. Upon exposure to dehydration, EL of the Spm-treated samples showed small change until 3 h, after which it rose to 51.2% at 12 h (Figure 1B). EL of the control remained stable 1 h after dehydration, followed by a noticeable increase at 3 h (58.3%) and 12 h (69.8%). At any time point after initiation of dehydration, water loss and EL of the control plants were significantly higher than those of the Spm-treated ones, implying that pretreatment with Spm enhanced dehydration tolerance.

Histochemical staining of Spm-treated and control plants under dehydration

Histochemical staining was employed to reveal in situ accumulation of O_2^- and H_2O_2, two important representatives for ROS. A short-term dehydration of less than 3 h did not produce detectable difference between leaves sampled from the control and Spm-treated plants. However, conspicuous difference was observed in the leaves at the last sampling time, in which Spm-treated leaves had less local blue spots (Figure 2A, indicator of O_2^-) and brown spots (Figure 2B, indicator of H_2O_2) than the control, suggesting that Spm treatment prior to dehydration decreased the accumulation of both O_2^- and H_2O_2.

Enzyme activity of Spm-treated and control plants under dehydration

Activities of SOD and POD were evaluated to examine the effect of Spm pretreatment on the antioxidant enzymes under dehydration. SOD activity of the control plants showed slight but continuous decrease during the whole course of dehydration. The Spm-treated plantlets had nearly the same SOD activity as the control during the first 3 h, while it exhibited a small rise and was significantly higher than the control at 12 h (Figure 3A). At the beginning of the dehydration stress, POD activity of the control plantlets was slightly lower than that of the Spm-treated ones. During the 12-h dehydration, POD activity was increased by 43.8% for the control and 41.0% for the Spm-treated plants (Figure 3B). As a result, at any time point the Spm-treated samples had higher POD activity than the control.

Endogenous free polyamines and expression of polyamine biosynthetic genes of Spm-treated and control plants under dehydration

The purport of this work was that exogenous application of Spm can modify the endogenous polyamine content. It is thus necessary to know if this truly happened. In this context, we measured

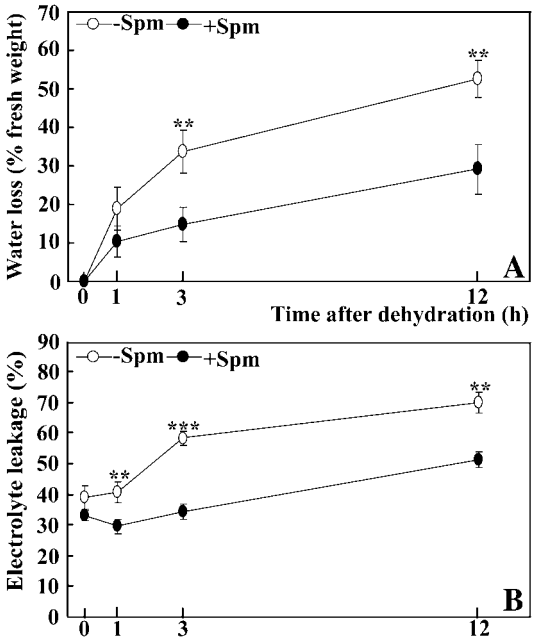

Figure 1. Water loss (A) and EL (B) of 'Red Tangerine' in vitro plants under 12-h dehydration, with (+Spm) or without (−Spm) pretreatment of 1 mM Spm. Asterisks show that the values are significantly different compared with the control (**$P < 0.01$ and ***$P < 0.001$).

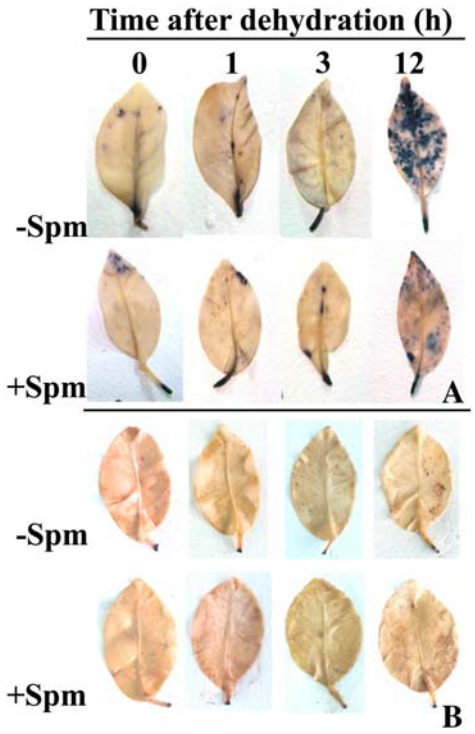

Figure 2. Histochemical staining assay of O_2^- (A) and H_2O_2 (B) by NBT and DAB, respectively.

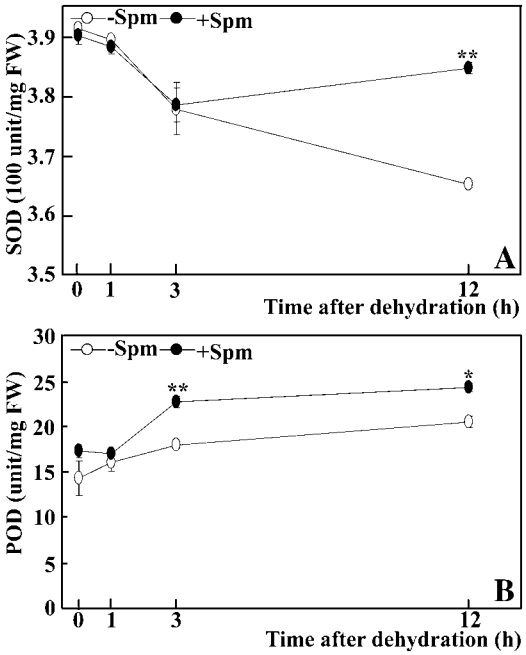

Figure 3. SOD and POD activities of 'Red Tangerine' in vitro plants under 12-h dehydration, with (+Spm) or without (−Spm) pretreatment of 1 mM Spm. Asterisks show that the values are significantly different compared with the control (*$P < 0.05$ and **$P < 0.01$).

the endogenous cellular polyamine content of the plants treated with or without Spm before and after dehydration. Exogenous treatment of Spm for 5 days led to a significant increase in free Spm, coupled with a small augmentation of Put, while Spd was not changed (0 h data in Figure 4). Upon exposure to dehydration, free Put of the Spm-treated plantlets slowly increased until 3 h, followed by a sharp increase at 12 h, which is nearly 15 times the content at 0 h. By contrast, the control plants experienced no change in Put content (Figure 4A). Under dehydration, free Spd of the control plants did not change markedly despite a 1.7-fold increase at the last time point compared with the level at the onset of dehydration (166.0 versus 99.7 nmol g^{-1}FW). Free Spd of the Spm-treated plants showed a progressive increase until 12 h, where the level was five times higher in comparison with that at 0 h (Figure 4B). Free Spm of both control and Spm-treated plantlets increased from the start of dehydration to the end of the experiment. No difference was detected at 1 and 3 h, whereas the Spm-pretreated samples had significantly higher Spm than the control at 12 h (415.6 nmol g^{-1}FW versus 236.4 nmol g^{-1}FW, Figure 4C). During the course of dehydration, the Spm-treated plants had higher polyamine content than the control, with the exception of Spm at 1 h. As transcriptional regulation is responsible for polyamine synthesis, expression of the polyamine biosynthetic genes (*ADC*, *ODC*, *SPDS*, *SPMS* and *SAMDC*) was assessed via RT–PCR using the samples harvested at 0, 1 and 12 h after dehydration (Figure 4D). Except *SPDS*, the other four genes exhibited detectable expression, although *ODC* signal was very weak. Before dehydration treatment, no conspicuous difference in the abundance of the genes was observed regardless of the Spm treatment. Under dehydration, *ADC* was abundantly induced in the Spm-treated samples at 1 h, followed by a decline at 12 h, which was still higher than that at 0 h. *ADC* mRNA level of the control was also induced at 1 h, but it was markedly lower compared with the Spm-treated ones. Steady-state level of *SPMS* was continuously up-regulated in the Spm-treated samples until 12 h, whereas that of the control showed reduction, yielding a lower expression level at the same time point in comparison with the Spm-treated samples. *SAMDC* expression was slightly induced at 12 h in both types of plant, but no obvious difference was detected between the two types of sample.

Stomatal response of Spm-treated and control plantlets to dehydration

As mentioned above (Figure 1A), Spm-treated plantlets lost less water than the control when they were dehydrated in the same situation. As water loss is primarily regulated by

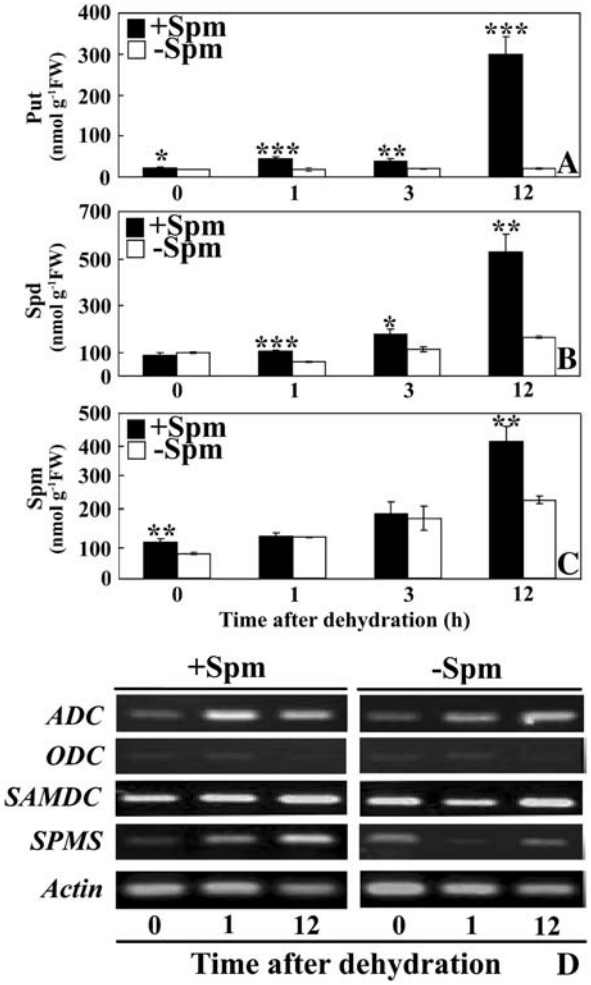

Figure 4. Endogenous free polyamine (A, Put; B, Spd; C, Spm) content and expression of biosynthetic genes (D) of 'Red Tangerine' in vitro plants under 12-h dehydration, with (+Spm) or without (−Spm) pretreatment of 1 mM Spm. Asterisks show that the values are significantly different compared with the control (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

stomata, stomatal apertures of samples collected at 0 and 12 h were then examined via SEM. Stomatal opening of the Spm-treated samples at 0 h was remarkably and significantly smaller than the control. After exposure to dehydration treatment for 12 h, stomata from both groups of plants displayed closure, whereas stomatal pores in the Spm-pretreated peels were still smaller than the control (Figure 5A and B). Stomatal width of the two types of sample was correlated with their water loss, implying different stomatal response between the Spm-treated and control plants.

Discussion

Effect of exogenous polyamine on enhancement of stress tolerance

It has been well known that polyamines are polycationic compounds at physiological pH, which is important to mediate their biological activity (Martin-Tanguy 2001). The property of binding several negatively charged molecules and thus stabilizing membrane have made them desirable targets to be employed for stress relief. In this research, Spm pretreatment conferred dehydration tolerance, corroborating previous work where exogenous polyamine application played a protective role against stress (Liu et al. 2007, Yamaguchi et al. 2007). The scenario of applying Spm, a tetramine, was that it contained higher net charge due to possession of four nitrogen groups, rendering greater buffering capacity relative to Spd and Put. Our results were in agreement with earlier work by Velikova et al. (1998, 2000) and Farooq et al. (2009). Nevertheless, it has to be mentioned that the role of individual polyamines in defense against stress still remains a matter of controversy, and a conclusive decision cannot be made at this point since contrasting results have been reported. For example, Yiu et al. (2009) reported that even though Spd and Spm effectively maintained water content under flooding, the former had better effect than the latter. A similar result has been obtained by Kurepa et al. (1998), who reported that Spd feeding offered the highest level of protection against paraquat toxicity. In another work, Tang and Newton (2005) demonstrated that Put was more effective for reducing salt-induced damage in Virginia pine. The discrepancy of these reports may be, at least in part, ascribed to difference in plant species/genotypes, physiological status and type of tissue used for experiment setup, polyamine solution concentration, treatment duration and experiment conditions (Iqbal and Ashraf 2005).

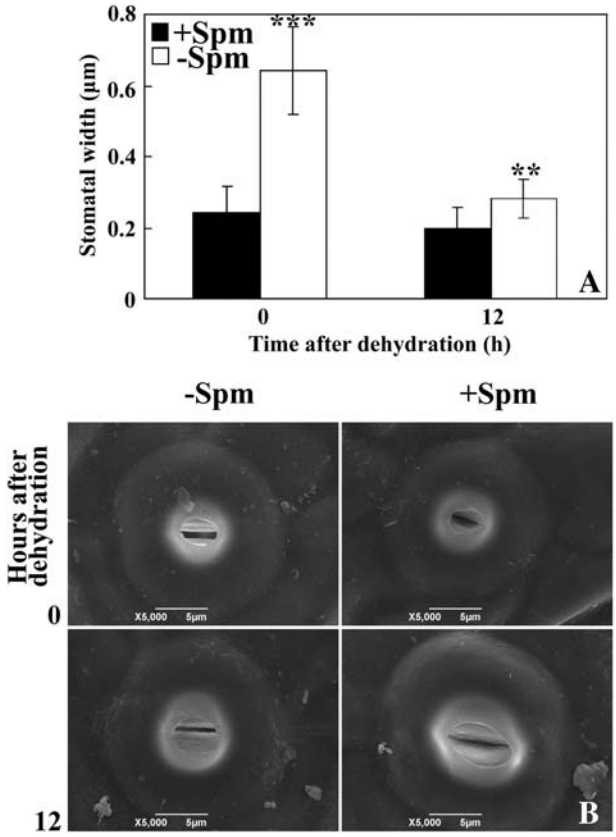

Figure 5. Comparison of stomatal aperture between 'Red Tangerine' epidermal peels sampled from in vitro plants at 0 and 12 h after dehydration, with (+Spm) or without (−Spm) pretreatment of 1 mM Spm. (A) Stomatal width of aperture size. (B) Representative SEM photographs showing the difference, taken at 5000-fold magnification. Asterisks show that the values are significantly different compared with the control (**$P < 0.01$ and ***$P < 0.001$).

Pretreatment of Spm led to higher endogenous polyamine content and reduced production of ROS

Spm treatment led to an expected increase in endogenous Spm content. Meanwhile, it was noted that endogenous Put was also slightly but significantly enhanced by pretreatment with Spm, implying that the cellular polyamine content may be subjected to a subtle regulation. Polyamine measurement showed that although both the control and Spm-treated plants increased endogenous polyamines under dehydration (with the exception of Put in the control), the Spm-treated ones had higher free polyamines than the control, in particular at the last time point (Figure 4). This result agrees well with that of Kubiś (2008), indicating that the exogenous application of polyamine had a positive effect on its endogenous counterpart. The higher endogenous polyamine content in the Spm-treated samples under dehydration suggests that there might be a feedback regulation in polyamine biosynthesis due to the pre-existing difference in free polyamine at 0 h (Liu et al. 2009). In addition, the possibility that greater induction of the polyamine biosynthetic genes such as *ADC* and *SPMS* by the higher polyamine content at 0 h could not be fully excluded, as evidenced from Figure 4D, although an intimate correlation between gene expression and polyamine content was not established, particularly for *ODC* and *SPDS*. Incongruence between gene expression and polyamine biosynthesis is assumed to be common as such a phenomenon was frequently reported when they were analyzed together (Liu and Moriguchi 2007, García-Jiménez et al. 2009). It

suggested that regulation of polyamine biosynthesis is not limited to transcriptional level, and regulation at other levels (post-transcriptional and translational) may be also implicated. In addition, absence of consistency between *ODC* gene expression and the polyamine level implied that the enzyme it encodes is primarily involved in cell division rather than in stress response (Kaur-Sawhney et al. 2003), while no detection of *SPDS* gene level raised a question concerning the presence of other *SPDS* gene isoforms that may be more closely linked to stress adaptation. On the other hand, polyamine catabolism and conjugation might also be taken into consideration when cellular polyamine biosynthesis is assessed as both of them are responsible for the regulation of cellular endogenous polyamine concentrations (Martin-Tanguy 2001, Paschalidis and Roubelakis-Angelakis 2005, Kusano et al. 2008). Unfortunately, we could not clarify the expression of genes involved in this pathway as relevant information is lacking in *Citrus* at this point. Nevertheless, as cellular polyamine content is influenced by many factors, the exact mechanism underlying the observed change herein is worth investigating in the future.

Histochemical staining revealed that the Spm-treated leaves contained remarkably less ROS than the control despite the absence of detectable difference between them within the first 3 h, suggesting a substantial decrease in the stress-raised ROS accumulation in the former. Interestingly, the decrease in ROS production was concurrent with the significantly higher free polyamines at 12 h in the Spm-treated plantlets, which makes it tempting to speculate that the accumulation of endogenous polyamines below a toxic level might contribute to the ROS removal. However, it should be noted that in our research we could not decipher which endogenous polyamine plays the exclusive or major role in dehydration tolerance. The use of specific polyamine biosynthetic inhibitors will possibly shed light on this issue.

Inhibition of ROS formation after Spm pretreatment was in accordance with previous work (Velikova et al. 2000, Zhao and Yang 2008, Yiu et al. 2009), supporting the widely accepted role of polyamines in ROS scavenging (Drolet et al. 1986, Bors et al. 1989, Ha et al. 1998). It is known that ROS formation requires an electron donation, which can be provided by metal ions, such as Fe^{2+} and Cu^{+} (Blokhina et al. 2003). Spm has been shown to form a ternary complex with iron and the phospholipid polar head, which impedes autoxidation of Fe and subsequently reduces generation of ROS (Tadolini 1988). This may responsible for less production of ROS after exogenous application of Spm before exertion of a dehydration treatment.

Pretreatment of Spm moderated the activities of antioxidant enzymes

In biological systems, formation of ROS is an important physiological process under adverse environments. As ROS are detrimental to cellular components, they have to be scavenged so as to maintain normal growth. Although the above-mentioned depiction showed that polyamines may directly interact with the ROS, it has to be mentioned that this effect may constitute only a partial defense mechanism against the excessive production of ROS. As an indispensible strategy for overcoming ROS attack, plants deploy detoxifying enzymes to control the ROS level, preventing the cells from oxidative stress (Arbona and Gómez-Cadenas 2008). Of the detoxifying enzymes, SOD plays an essential protective role in scavenging ROS as it is the first line of defense against ROS. H_2O_2 produced via SOD action is further scavenged by POD, along with catalase and ascorbate peroxidase (Jaleel et al. 2009). Activities of SOD and POD were analyzed to get insights into the oxidative status of the dehydrated plants with or without pretreatment with Spm. It can be seen that under dehydration SOD activity of the Spm-treated plants was significantly higher than the control at the last point despite no difference from 0 to 3 h. Decrease in SOD activity under stress has been reported in previous experiments (Groppa et al. 2001, Kim and Jin 2006, Kubiś 2008, Yiu et al. 2009). The significant increase in SOD at 12 h in the Spm-treated samples implied that the typical stress-induced decline in the total activity of SOD has been prevented at the end of the dehydration, in line with a previous report (Kim and Jin 2006). Different from SOD, POD activity was increased in the dehydrated plants irrespective of Spm pretreatment. However, it is clear that at any time point POD activity in the plants pretreated with Spm was higher than the control.

The increased antioxidant enzyme activities were largely associated with enhanced polyamine accumulation under stressful condition. Our data, along with others via either exogenous polyamine addition or genetic transformation of polyamine biosynthetic genes, have collectively shown that polyamines can possibly moderate the activities of antioxidant enzymes under stress (Groppa et al. 2001, Kim and Jin 2006, Wi et al. 2006, He et al. 2008, Kubiś 2008, Wen et al. 2009, Yiu et al. 2009). Higher induction of the enzymes may contribute to the effective removal of stress-raised ROS, leading to less oxidative stress and better stress tolerance. For instance, the rise of SOD level at 12 h was perfectly consistent with less in situ staining of O_2^{-}. This can, at least partially, explain the lower ROS formation in Spm-treated leaves under dehydration in comparison with the control. However, it remains unclear how polyamines can influence the function of antioxidant enzymes. One possible reason is that polyamines stimulate de novo synthesis of the antioxidant enzymes at the translational level (Kim and Jin 2006).

Different stomatal response to dehydration in Spm-treated and control plants

The Spm-treated plants showed less wilted phenotype (data not shown) than the control under dehydration, coupled with significantly lower water loss. As water loss is primarily the result of stomata-mediated evaporation, the stomatal aperture at the onset and end of dehydration was thus com-

pared between the two types of leaves. It clearly showed that, in accordance with less water loss under dehydration, the Spm-treated leaves exhibited smaller stomatal aperture than the control at both time points, suggesting that polyamines might be involved in stomatal movement under dehydration. Our work corroborated earlier work done on other plants, such as *Vicia faba* and *A. thaliana* (Liu et al. 2000, Yamaguchi et al. 2007). Although we could not decipher the mechanism underlying the role of polyamines in stomatal response in this work, stomatal regulation by polyamine has been previously investigated. The intracellular polyamines have been shown to inhibit the voltage-dependent inward K^+ channel in the plasma membrane of guard cells and thus modulated stomatal aperture (Liu et al. 2000). Recently, Yamaguchi et al. (2007) proposed that accumulation of polyamines under dehydration increased cytoplasmic Ca^{2+} content due to modulation of Ca^{2+}-permeable channels, which subsequently inactivated the K^+-inward rectifier, leading to stimulated stomatal closure. Therefore, we can hypothesize that exogenously applied Spm resulted in higher accumulation of endogenous polyamine pool, which then takes part in or activates a cellular signaling pathway related to stomatal movement. The higher polyamines may stimulate stomatal closure under dehydration stress, leading to decreased water evaporation, which may be the scenario linking polyamine accumulation and less water loss. However, it is noted that the difference in the stomatal width between the Spm-treated and the control leaves was more apparent at 0 h than at 12 h after dehydration, which is not positively correlated with the change in polyamine content of the two time points. One possibility for this inconsistency is that the stomatal response might be effectively and subtly regulated by even low content of polyamines.

Taken together, our work showed that in vitro plants that are pretreated with exogenous Spm were more tolerant to dehydration stress than those without Spm prefeeding, as is shown by less water loss and lower EL. The enhanced tolerance was accompanied by higher polyamine content. The decreased water loss may be ascribed to inhibition of water evaporation via promotion of stomatal closure. Meanwhile, lower EL might be due to inhibition of lipid peroxidation and stabilization of biomembranes through direct ROS scavenging or indirect effect via activation of antioxidant enzymes. These two aspects, mediated by polyamines, may function together to confer the dehydration tolerance. All of these data suggested that polyamines, at least Spm herein, may be used as efficient protectors for abatement of stress-induced damage.

Acknowledgments

The current work is financially co-supported by projects from National Natural Science Foundation of China (30871685, 30921002), 2006BAD01A1702, the Ministry of Education of China (NCET-06-0666) and Hubei Provincial Natural Science Foundation (2009CDA080).

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