



The alterations of endogenous polyamines and phytohormones induced by exogenous application of spermidine regulate antioxidant metabolism, metallothionein and relevant genes conferring drought tolerance in white clover



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ABSTRACT

Cross-talk between polyamines (PAs) and phytohormones may be involved in the regulation of drought tolerance in plants. The objective of this study was to determine the effects of exogenous spermidine (Spd) and a Spd inhibitor, dimethylthiourea (DCHA), on drought responses of white clover (*Trifolium repens*). Metabolic, protein, and gene transcript levels were evaluated at leaf relative water content levels of 87% for non-stressed plants and 50% for drought-stressed plants. The exogenous application of Spd (0.05 mM) significantly increased endogenous PAs content whereas DCHA generally reduced PAs content. Phytohormone responses to Spd application included an elevated gibberellic acid (GA) and indole-3-acetic acid (IAA) content under non-stressed condition, and an increased GA and cytokinin (CTK) content as well as decreased IAA content under water deficit condition. The increase in endogenous PAs had no significant effect on abscisic acid (ABA) accumulation under either water treatment. Spd treatment resulted in enhanced antioxidant defense via maintenance of greater antioxidant enzyme activities and transcript levels, especially ascorbic acid (AsA). Other metabolites such as total phenols, flavonoids, proline, metallothionein (MT), and cysteine (Cyt) were also elevated with Spd treatment. On the contrary, inhibition of Spd biosynthesis weakened antioxidant defense, decreased proline metabolism, and inhibited MT genes expression leading to aggravation of drought-induced stress damages. These results suggest that changes of PAs and phytohormones play critical roles in the improvement of drought tolerance in white clover, which are attributed to the alleviation of oxidative stress, inhibition of leaf senescence, and maintenance of growth under drought stress.

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Abbreviations: ABA, abscisic acid; APX, ascorbate peroxidase; AsA–GSH cycle, ascorbate–glutathione cycle; AsA, ascorbic acid; CAT, catalase; Chl, chlorophyll; CTK, cytokinin; Cyt, cysteine; DAsA, dehydroascorbic acid; DCHA, dimethylthiourea; DHAR, dehydroascorbate reductase; EL, electrolyte leakage; ELISA, enzyme linked immunosorbent assay; GA, gibberellic acid; GPOX, guaiacol peroxidase; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, glutathione disulfide; GST, glutathione S-transferase; H₂O₂, hydrogen peroxide; HPLC, high performance liquid chromatography; IAA, indole-3-acetic acid; LSD, least significance difference; MDA, malondialdehyde; MDHR, monodehydroascorbate reductase; MT, metallothionein; O₂^{•-}, superoxide anion radical; OAT, ornithine aminotransferase; P5CS, Δ^1 -pyrroline-5-carboxylate synthetase; PAs, polyamines; PEG, polyethylene glycol; ProDH, proline dehydrogenase; Put, Putrescine; qRT-PCR, real-time quantitative polymerase chain reaction; RGR, relative growth rate; ROS, reactive oxygen species; RWC, relative water content; SOD, superoxide dismutase; Spd, spermidine; Spm, spermine.

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1. Introduction

Water deficit limits crop yield and quality worldwide, especially in cool-season perennial forage species such as white clover [*Trifolium repens* (Annicchiarico and Piano, 2004)]. Investigation of metabolites and mechanisms in plants to better understand how we may improve drought tolerance of plants is desirable in agriculture. Low molecular weight nitrogen-containing compounds known as polyamines (PAs) have been found to play an important role in plant growth regulation. PAs have multiple biochemical and physiological function related to the response to environmental stresses (Kaur-Sawhney et al., 2003; Liu et al., 2007). Putrescine (Put), spermidine (Spd), and spermine (Spm) are the most common and abundant PAs in plants, since they respond to high temperature (Gonzalez-Aguilar et al., 1997), drought

(Yamaguchi et al., 2007), and salinity (Roychoudhury et al., 2011; Tanou et al., 2014) stress. Drought-tolerant plants generally increase endogenous PAs content or metabolism to higher levels than sensitive plants in response to drought stress (Yang et al., 2007; Alcázar et al., 2011). In addition, exogenous application of PAs enhanced drought tolerance of cucumber [*Cucumis sativus* (Kubiš, 2008)], rice [*Oryza sativa* (Farooq et al., 2009)], dwarf cherry [*Prunus cerasus* (Yin et al., 2014)], and creeping bentgrass [*Agrostis stolonifera* (Shukla et al., 2015)]. Inhibition of PAs biosynthesis amplified stress-induced damage in pear [*Pyrus communis* (Wen et al., 2011)]. However, the role of PAs on improving stress tolerance has not yet been fully elucidated.

How PAs may be associated with other signaling agents in plants like phytohormones and stress protective compounds, such as flavonoids, could help elucidate the induced defenses observed due to PAs application or endogenous regulation. In general, how phytohormones respond to drought stress and how they may interact with each other is known for many plant species (Wang et al., 2011; Yuan et al., 2012; Krishnan and Merewitz, 2015). Drought triggers abscisic acid (ABA) accumulation in plants to induce stomatal closure, thereby reducing water loss (Gibson et al., 1991; Kholova et al., 2010; Morkunas et al., 2014). Different from ABA, the physiological function of CTK mainly is to delay leaf senescence in response to drought stress (Rivero et al., 2007; Merewitz et al., 2010). An increase in endogenous CTK content has been found to be effective in improving drought tolerance in different plant species via the exogenous application of CTK or a transgenic approach using overexpression of a CTK biosynthesis gene (Clark et al., 2004; Peleg et al., 2011; Merewitz et al., 2011a). In contrast, the roles of gibberellic acid (GA) and IAA in drought tolerance are very contradictory and may be species dependent (Yang et al., 2001; Zhang et al., 2009; Dobra et al., 2010; Rodrigues et al., 2011). It has been demonstrated that foliar application of Spd and Spm significantly increased endogenous PAs, indole-3-acetic acid (IAA), abscisic acid (ABA), and cytokinin (CTK) content during wheat grain filling [*Triticum aestivum* (Liu et al., 2013)]. Exogenous ABA increased Put accumulation in embryonic axes of chickpeas (*Cicer arietinum*) under non-stressed conditions (Bueno and Matilla, 1992) and the decrease of ABA content by using an ABA biosynthesis inhibitor reduced the accumulation of PAs in salt-stressed maize leaves [*Zea mays* (Liu et al., 2005)]. It seems clear that PAs play a role in drought tolerance in plants and may interact with other phytohormones. Whether the cross-talk between PAs and phytohormones is important for drought tolerance in plants is not well known.

The protective role of secondary metabolites such as proline, MT, and flavonoids during stress are well-known but whether PAs are involved in the regulation of these compounds is still unclear in plants. It has been demonstrated that drought stress causes a significant generation of ROS leading to lipid peroxidation and damage to plant cells (Hasanuzzaman et al., 2012). Ascorbate-glutathione cycle (AsA–GSH cycle) plays a vital role in the antioxidant system of plants. Enzymes involved in the AsA–GSH cycle directly scavenge H₂O₂ as do the products of the pathway, AsA and GSH (Li et al., 2010). Apart from antioxidant enzymes, some secondary metabolites in plants also serve as free radical scavengers (Hagerman et al., 2003; Aron and Kennedy, 2008). Protective phenol compounds such as flavonoids also play a significant role in ROS scavenging in plants. Flavonoids catalyze oxygenation reactions that serve to scavenge ROS and contribute to drought tolerance (Król et al., 2014; Nakabayashi et al., 2014a). For example, proanthocyanidins belong to the flavonoids family and have strong antioxidant capacities (Wu et al., 2004; Gonçalves et al., 2005). Proline also plays an important role in stress tolerance in plants, since it has two primary protective functions in response to different environmental stresses: one is

to modulate osmotic potential together with compatible solutes and the other is to help to maintain redox balance and scavenge ROS acting as a singlet oxygen quencher (Szabados and Savoure, 2010). Another important metabolite in drought stress is metallothionein (MT). MT is a small cysteine-rich protein and responds to various abiotic stresses in plants, especially heavy metal stress, salt stress, and drought stress (Kawasaki et al., 2001; Moloudi et al., 2013; Liu et al., 2014). In spite of these facts, the interaction of PA levels with phytohormones content, antioxidants, and other metabolites associated with drought tolerance deserves further investigation.

Previous studies have indicated that PAs can improve drought tolerance through involvement in osmotic adjustment, maintaining a cation–anion balance, or antioxidant defense in plants (Bouchereau et al., 1999; Alcázar et al., 2010). Limited information is still available on the knowledge of how PAs regulate such tolerance mechanisms. An investigation how PAs may be associated with phytohormones, proteins, and the expression of genes related to drought tolerance is needed. In addition, many studies have focused on revealing the functions of PAs under different drought durations (Kasukabe et al., 2004; Farooq et al., 2009; Shi et al., 2010; Yin et al., 2014), but it is still not clear how PAs-regulated responses are altered when plants are evaluated at the same level of water deficit. Therefore, the objectives of this study were to analyze the interaction between the drought stress and elevated or declined endogenous PAs induced by exogenous application of Spd and DCHA, an inhibitor of biosynthesis, on phytohormone levels, the ASA–GAH cycle, MT and the expression of relevant genes, proline metabolism.

2. Materials and methods

2.1. Plant materials and treatments

Seeds of white clover (cv. Ladino) were sterilized with 0.1% mercuric chloride for 4 min and rinsed 3 times in distilled water. Seeds (0.1 g) were sown in trays (24 cm length, 20 cm width and 15 cm deep) filled with sterilized quartz sand and distilled water, and then put in a controlled growth chamber set at 12 h photoperiod, 75% relative humidity, 23/19 °C day/night temperature, and 500 μmol m⁻² s⁻¹ photosynthetically active radiation. After 7 d of germination, the distilled water in tray was replaced by full-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950) and plants were cultivated for another 23 d (replacing the solution every other day). Drought was induced by polyethylene glycol (PEG) 6000 dissolved in Hoagland's solution in this study. PEG solution osmotic potential was kept –0.5 MPa (Vapro Pressure Osmometer, Wescor, Inc., Logan, UT 84321, USA). Plants were subjected to six treatments: (1) well-watered control; (2) well-watered plants treated with 0.05 mM spermidine (Spd); (3) well-watered plants treated with 2 mM dimethylthiourea (DCHA), an inhibitor of PAs biosynthesis; (4) drought; (5) drought-stressed plants treated with 0.05 mM Spd; (6) drought-stressed plants treated with 2 mM DCHA. Plants were pretreated with 2 mM DCHA or 0.05 mM Spd nutrient solution for 3 d before stress. All treatments were performed randomly on four individual plants. For each measurement, four samples (replicates) were taken, one from each individual plant. When the relative water content (RWC) of leaves declined to 49–51% (no statistically significant difference) for all plant treatments, plant samples were taken for all the measurements described below. This meant that on day 5 of drought stress plants treated with DCHA were sampled (50.6% RWC), on day 6 drought-stressed control plants were sampled (48.9% RWC), and on day 7 samples were collected for drought-stressed plants treated with Spd (51.1% RWC). The second leaf on several stems from each plant were harvested for all analyses in

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