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Research Paper

Na$^+$ induces the tolerance to water stress in white clover associated with osmotic adjustment and aquaporins-mediated water transport and balance in root and leaf

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ABSTRACT

Aquapotins (AQPs) could be involved in Na$^+$-regulated tolerance to water stress in white clover. White clovers were subjected to water stress (−0.3 MPa) induced by polyethylene glycol 8000 for 16 days in the presence or in absence of NaCl (30 mM) pretreatment. Na$^+$ pretreatment significantly enhanced the tolerance to water stress in white clovers based on growth and physiological analyses. Na$^+$-induced increases in endogenous Na$^+$ content and transcript levels of *NHX6*, *SOS1*, and *SKOR* associated with ions compartmentalization could be responsible for higher osmotic adjustment (OA) as opposed to the accumulation of more organic osmolytes (proline and water soluble carbohydrates) in leaf and root. Na$^+$ pretreatment not only further elevated endogenous ABA content caused by water deficit, but also enhanced the accumulation of AQPs and the expression of genes encoding AQPs under water stress. The increase in ABA content induced by Na$^+$ could contribute to the enhancement of ABA-activated stress defense in relation to transcription factors and AQPs in root and leaf. Na$^+$-treated white clover exhibited significantly greater root growth and OA in root and leaf, but the significant decline in transpiration rate in leaf under water stress, which provided a beneficial foundation for the AQPs-regulated water transport leading to a favourable water status in white clover under water stress. These results indicated that Na$^+$ could play a positive role in activating ABA-regulated AQPs in response to water deficit in mesophytic plants.

1. Introduction

The role of Na$^+$ in plants exposed to salt stress and the potentially toxic effect of Na$^+$ accumulation have been extensively studied. A large amount of Na$^+$ in soil can decrease soil water potential leading to the limitation of the water uptake in plant roots. In addition, the over-accumulation of Na$^+$ in cells not only cause ionic toxicity and ion leakage, but also could disrupt the absorption of other irons resulting in nutrition imbalance in plants (Hasegawa et al., 2000; Kawa et al., 2016; Zhu, 2003). Despite these negative effects, increasing evidence has demonstrated that plants could acquire stress tolerance after a short-term exposure to relatively low Na$^+$ (Glenn and Brown, 1998; Martínez et al., 2005; Saha et al., 2010). Na$^+$ in cells was transported into vacuole to maintain osmotic adjustment (OA), which is an important and common regulatory mechanism of plants in response to water deficit and salt stress (Hui and Jiang, 2010). Song et al. (2006) found that the contribution of Na$^+$ to water potential was over 50% in halophytic species *Suaeda physophora* and *Haloxylon ammodendron* under field condition and arid environment. Na$^+$ accumulation rather than exclusion in succulent xerophytes *Haloxylon ammodendron* and *Zygophyllum xanthoxylum* could be one of the most effective strategies for the adaptation to drought stress (Wang et al., 2004). The study of Glenn and Brown (1998) has also showed that an appropriate amount of Na$^+$ uptake in *Atriplex canescens* could enhance the ability to survive

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Abbreviations: ABA, abscisic acid; AQPs, aquapotins; Chl, chlorophyll; CTK, cytokinin; EL, electrolyte leakage; GA, gibberellic acid; IAA, indole-3-acetic acid; LSD, least significance difference; MDA, malondialdehyde; Mg-CHT, Mg-chelatase; NIPs, nodulin 26-like intrinsic proteins; OA, osmotic adjustment; PAO, pheophorbide a oxygenase; PIPs, plasma membrane intrinsic proteins; PBGD, porphobilinogen deaminase; PEG, polyethylene glycol; Pn, net photosynthetic rate; POR, protochlorophyllide reductase; qRT-PCR, real-time quantitative polymerase chain reaction; RGR, relative growth rate; RWC, relative water content; SIPs, small intrinsic proteins; TIPs, tonoplast intrinsic proteins; Tr, transpiration rate; WUE, water use efficiency; WSC, water soluble carbohydrates

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drought stress. In absence of K^+, Na^+ could replace K^+ as a critical inorganic solute in vacuoles for the maintenance of osmotic potential and also could alleviate the symptoms of K^+ deficiency in plants (Subbarao et al., 2003). *Zea mays* plants after exposed to low level salinity could achieve a significant improvement of salt tolerance because the salt-pretreated plants retained more K^+ in roots and enhanced vacuolar Na^+ sequestration ability in leaves contributing to better OA in shoots (Pandolfi et al., 2016). In spite of these previous findings, further understanding of Na^+-induced tolerance to water stress is imperative for mesophytic crops adapting to various abiotic stresses.

Plants tolerance to water deficit involves complex traits including phytohormones regulation. Endogenous hormonal metabolism and balance are known to be highly related to whole-plant stress responses (Vanstraelen and Benková, 2012). Abscisic acid (ABA) accumulation induces stomatal closure resulting in the decrease in transpiration and water loss, which is the primary line of drought defense in plants (Wilkinson and Davies, 2002). Addition to the regulation of stomatal closure, ABA promotes antioxidant defense to detoxify reactive oxygen species (ROS) damage or triggers transcription factors to induce stress-related genes expression closely associated with enhanced drought tolerance in plants (Cutler et al., 2010; Lu et al., 2009). Cytokinin (CTK) in plants could serve a positive regulator in alleviating stress-induced senescence (Choi and Hwang, 2007; Lim et al., 2003). CTK-activated antioxidant and carbohydrates metabolism could decrease water deficit-induced cellular oxidative damage and also can improve photosynthesis and osmotic OA contributing to better tolerance when plants respond to water deficit (Merewitz et al., 2012, 2011a,b). Gibberellin (GA) and auxin indole-3-acetic acid (IAA) are previously in relation to plants growth (Vanstraelen and Benková, 2012). Differently, roles of GA and IAA in regulating drought tolerance often show contradictory in plants. The relationship between these two hormones and drought tolerance depends on plant species, tissue maturity, and stress duration and intensity (Li et al., 2016; Xing et al., 2016; Zawaski and Busov, 2014; Zhang et al., 2009). The complexity of stress mechanisms suggests that crops can well adapt to water deficit depending on the cross talk among different hormones (Peleg and Blumwald, 2011; Vanstraelen and Benková, 2012). Previous studies have proved that the alleviation of salt damage in plants is associated with changes of endogenous hormones (Javid et al., 2011; Ma et al., 2016). However, whether positive effects of the pretreatment with Na^+ have a correlation with hormones regulation from root to leaf under water stress still deserves further investigation.

The tolerance to water stress regulated by ABA and CTK is also related to proteins synthesis in plants, such as dehydrins, metallothionein, and aquaporins (AQPs) (Cerny et al., 2011; Li et al., 2016; Shatil-Cohen et al., 2011; Wang et al., 2002). AQPs are a class of intrinsic membrane proteins that exist in the plasma membrane and most of cell inner membranes regulating a wide range of water, CO_2, and other small neutral molecules (H_2O_2, silicic acid, and arsenite) transport in animals and plants under normal and stress conditions (Chrispeels and Agre, 1994; Groszmann et al., 2017; Maurel et al., 2015). It has been found that plant AQPs are mainly divided into 4 different subclasses including plasma membrane intrinsic proteins (PIPs), small intrinsic proteins (SIPs), tonoplast intrinsic proteins (TIPs), and nodulin 26-like intrinsic proteins (NIPs) (Maurel et al., 2015). These AQPs, known as water channels, play critical roles in the regulation of cell-to-cell water movement through the transcellular path (Javot and Maurel, 2002). AOPs acting as ion channels have been studied in earlier study (Byrt et al., 2016). AQPs also showed other important functions in adjusting transpiration, photosynthesis, and shoot growth with respect to the improvement of plant performance and recovery from severe water stress (Maurel et al., 2016; Secchi et al., 2017). The study of Almeida-Rodriguez and Hacke (2012) has revealed that drought stress induced the expression of *PIP2;3*, *PIP2;5*, and *TIP2;1* in stems of poplars (*Populus*), which is an important and feasible way to regulate water exchange between apoplast and symplast. Improved drought and salt tolerance in *Arabidopsis* could be created by overexpressing a *MaPIP1;1* gene in relation to the regulation of primary root elongation, water uptake, and membrane stability (Xu et al., 2014). However, the overexpression of a *Arabidopsis* plasma membrane aquaporin *PIP1b* could significantly improve cell vigor and photosynthesis in tobacco (*Nicotiana tabacum*) under favourable growth conditions, whereas facilitated faster wilting under drought stress (Aharon et al., 2003). Therefore, functions of different AQPs still need to further investigation when plants are exposed to water stress. In addition, Na^+ directly or indirectly affects AQPs accumulation or expression in plants. For example, NaCl stress reduced the abundance of PIP2 in roots of *Arabidopsis*, but increased the expression of *FrPIP2;1* in leaf of red fescue (*Festuca rubra*) (Chen et al., 2005; Diédhiou et al., 2009). It has been reported that the promoter of *NtAQP1* includes ABA response element (CTAACCA), and the *NtAQP1* could be activated by ABA in tobacco (Siefritz et al., 2001). Many stress-mediated AQPs could also be regulated by ABA in plants (Ding et al., 2016a; Li et al., 2008). In spite of these studies, limited information is available about Na^+-regulated water transport and balance related to changes of AQPs and ABA in plants under water-limited condition.

White clover (*Trifolium repens*), an important legume forage, is widely cultivated all over the world and provides high quantity of crude proteins for animal nutrition, but sensitive to water deficit due to shallow root system and less adaptation to arid environment (Annicchiarico and Piano, 2004). With a progressive decline in available irrigation water worldwide, drought-induced limitations of growth, development, and productivity of crops will further expand (Cattivelli et al., 2008). In order to better understand the possible relationship between Na^+ accumulation and ABA-regulated AQPs in response to water deficit, the objectives of this study were to 1) examine whether appropriate Na^+ pretreatment could improve the tolerance to water stress in mesophytic white clover via analyses of growth and physiological changes; 2) explore whether OA and Na^+/K^+ transportation could be affected by Na^+ pretreatment under water stress; and 3) reveal whether alterations of phytohormones, stress-related genes, and aquaporins contributing to the regulation of water transport and balance could be involved in Na^+-induced tolerance to water stress in leaf and root.

2. Materials and methods

2.1. Plant material and treatment

Seeds of white clover cv.'Ladino' were surface sterilized using 0.1% mercuric chloride solution for 4 min and rinsed 3 times with distilled water. 0.05 g seeds were germinated in each plastic pot (24 cm length, 20 cm width, and 15 cm deep) filled with sterilized quartz sands in a controlled growth chamber (12 h photoperiod cycle, 23/19 °C day/night temperature, 730 μmol photons $m^{-2} s^{-1}$ photosynthetic active radiation (PAR), and a relative humidity of 75%). Seven-day-old seedlings were irrigated with Hoagland's solution (Hogland and Arnon, 1950) until the second leaves fully expanded. The thirty-day-old plants in the same size were carefully removed from quartz sands and then subjected to either 0 (control) or 30 mM sodium chloride (NaCl) in Hoagland's solution for 3 days as the pretreatment. The pretreated and untreated plants were subjected to Hoagland's solution (control) or water stress induced by polyethylene glycol (PEG) 8000 dissolving in Hoagland's solution for another 16 days, and osmotic potential of PEG solution was maintained at-0.3 MPa (Vapro pressure osmometer, Wescor, Inc. Logan, UT 84321, USA). The Hoagland's solution and PEG solution were refreshed every day to avoid the change of solution concentration and aerated through aeration pumps (115 V, 60 Hz, Tetra® Blacksburg, VA) to provide enough oxygen in solution. Hence, there were four different treatments: (1) C (water-sufficient control) (2) C + Na^+ (water-sufficient control pretreated with 30 mM NaCl); (3) D