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Research article

An unusual strategy of stomatal control in the desert shrub *Ammopiptanthus mongolicus*

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ABSTRACT

Water deficit is one of the main environmental constraints that limit plant growth. Accordingly, plants evoke rather complex strategies to respond and/or acclimate to such frustrating circumstances. Due to insufficient understandings of acclimatory mechanisms of plants' tolerance to persistent water deficit, a desert shrub of an ancient origin, *Ammopiptanthus mongolicus,* has recently attracted growing attentions. Differed from Arabidopsis, the opening of stomata of *A. mongolicus* is constrained by low external K^+ concentration of the guard cells. Although as a general consequence, a raised level of ABA is also induced in *A. mongolicus* following water deficit, this does not accordingly result in efficient stomatal closure. In consistent with this phenomenon, the expression of genes coding for the negative regulators of the ABA signaling cascade—the type 2C protein phosphatases (PP2Cs) are notably induced, whereas the transcription of the downstream SnRK2 protein kinase genes or the destination ion fluxing channel genes remain almost unaffected under water deficit treatments. Therefore, in term of stomatal control in response to water deficit, *A. mongolicus* seemingly employs an unusual strategy: a constrained stomatal opening controlled by extracellular K^+ concentrations rather than a prompt stomatal closure triggered by ABA-induced signaling pathway. Additionally, an acute accumulation of proline is induced by water deficit which may partly compromise the activation of antioxidant enzymes in *A. mongolicus*. Such strategy of stomatal control found in *A. mongolicus* may in certain extents, reflect the acclimatory divergence for plants' coping with persistent stress of water deficit.

1. Introduction

Water deficit is one of the most common environmental stresses that constraints the growth and development of plants (Bray, 1997; Fang and Xiong, 2015; Ma et al., 2016). Plants response to water deficit stress is a complex physiological, biochemical and molecular processes (Bhargava and Sawant, 2013). Numerous reports from herbaceous model plants, such as Arabidopsis (Seki et al., 2002; Hummel et al., 2010; Des Marais et al., 2012) and rice (Gorantla et al., 2007; Lima et al., 2015) have established insightful physiological, biochemical and molecular mechanisms responding to relatively 'instantaneous' drought stresses. However, strategies of drought stress resistance/response in woody plants, especially those suffering consistent threaten of water deficit stress, have not been sufficiently resolved.

Stomata are major passage for plant water transpiration and thus are often among the central concerns of plants' response to water deficit. It has been demonstrated in Arabidopsis, two closely related KAT channels dominate K^+ uptake into the guard cells and mediate the opening of the stomata (Lebaudy et al., 2008). Whereas the activation of the KAT channels is not regulated by the availability of external K^+ (Véry et al., 1995; Pilot et al., 2001; Yang et al., 2015a). On the opposite, a guard cell K^+ release channel GORK is responsible to control the closure of the stomata (Hosy et al., 2003). Within the well defined models of drought stress induced ABA signaling cascade, the activation of GORK channels is eventually recognized as major effectors leading to the closure of stomata (Li et al., 2006; Pandey et al., 2007; Joshi-Saha et al., 2011).

Under drought stresses, ABA is accumulated in plants as the result of enhanced activities of key enzymes, 1-deoxy-D-xylulose-5-phosphate synthase (DXS), zeaxanthin epoxidase (ZEP) and 9-cis-epoxycarotenoid

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dioxygenase (NCED) involved in the biosynthesis of the phytohormone (Seo and Koshiba, 2002). Upon the accumulation of ABA, the ABA signaling network initiates. Within this signaling cascade, the type 2C protein phosphatases (PP2Cs) act as the center component which form interactions with ABA receptors and the SnRK2 family kinases (Fujii et al., 2009; Umezawa et al., 2009). In the absence of ABA, a PP2C binds to the SnRK2 kinase and inactivates its kinase activity by dephosphorylating a serine residue in the kinase activation loop (Soon et al., 2012). Under stress conditions, raised level of ABA promotes the binding of PYL/PYR/RCAR family receptors to PP2Cs and inhibits the catalytic activity of PP2Cs which releases the activation of SnRK2s previously blocked by PP2C and allows the successive transduction of signals to downstream effectors (Park et al., 2009; Cutler et al., 2010; Soon et al., 2012). Such that the anion channels (for instance, SLACs) are activated upon successful phosphorylation whose activity depolarizes the plasma membrane potential of guard cells; then the out-ward rectifying K^+ channels (for instance, GORK) are activated, mediating release of K^+ from guard cells that eventually leads to the closure of the stomata (for reviews, see Joshi-Saha et al., 2011; Brandt et al., 2012; Munemasa et al., 2015).

In the well-defined signaling network of ABA-induced stomatal closure, PP2Cs play a central role but act as negative regulators in highly ABA-dependent manner (Umezawa et al., 2009; Vlad et al., 2009). However, elevated levels of *PP2C* gene transcripts following drought stress or ABA treatments have been repeatedly observed in many plant species (Saez et al., 2004; Chen et al., 2015; Xiang et al., 2017) that implies a compromised sensitivity of ABA signaling. For instance, overexpression of an *Arabidopsis PP2C* ortholog, *HAB1* led to a drought-hypersensitive/ABA-insensitive phenotype of the transgenic plants (Saez et al., 2004). Overexpression of a maize *PP2C* gene (*ZmPP2C*) in *Arabidopsis* decreased plant tolerance to drought and salt (Liu et al., 2009). The expression of a poplar *PP2C* ortholog (*PeHAB1*) was induced by drought and ABA treatments and *Arabidopsis* plants over-expressing this gene showed decreased ABA sensitivity and reduced tolerance to drought (Chen et al., 2015).

Drought stress usually causes over-accumulation of reactive oxygen species (ROS) in plants, which in turn activate the ROS scavenging enzymes, the so-called defense systems, such as CAT, POD, SOD and APX, to prevent plants from severe oxidative injury (Ahmad et al., 2008; Cruz de Carvalho, 2008).

Accumulation of soluble metabolites such as free amino acids and soluble sugars in leaves is a useful approach in many dry-land plants for increasing osmotic potential and constraining plant free water from severe transpirational loss (Chen and Jiang, 2010). Among these osmoregulation substances, proline is capable of enhancing drought tolerance in plants and is considered to act not only as an osmolyte, but also an effective ROS scavenger (Dobra et al., 2010). Overexpression of a key proline synthesis gene coding for delta-1-pyrroline-5-carboxylate synthase (P5CS) resulted in increased proline contents and enhanced drought stress tolerance in tobacco (Kishor et al., 1995; Hong et al., 2000) and petunia (Yamada et al., 2005). Soluble sugars can also accumulate in leaf cells to maintain the osmotic potential and protect plants from severe water loss (Krasensky and Jonak, 2012). In *Atriplexhalimus* L. (Martinez et al., 2005) and rubber tree (Wang, 2014), soluble sugars are considered as the main contributors to osmotic adjustment in response to drought stresses.

Photosynthesis is the primary metabolic process for carbon assimilation in plants. Under drought stress conditions, it is supposed that closed stomata could limit CO_2 assimilation and consequently reduces the photosynthetic rate (Chaves et al., 2009; Saibo et al., 2009; Ma et al., 2016). Accordingly growth reduction and/or alteration of carbohydrate metabolism often occur in plants suffering prolonged water deficit stresses. Such reduction in shoot biomass accumulation and altered carbohydrate metabolism are proposed as an active protection for plants' survival over prolonged water deficit (Tardieu et al., 2014). Even under the early stage of water deficit, before an apparent growth reduction being visualized, down-regulation of photosynthetic genes and enhanced expression of genes involved in the degradation of polymerized carbohydrates could be readily evidenced, indicating that plants are able to respond or acclimate to the water availability status sensitively and promptly (Nouri et al., 2015; Ma et al., 2016; Thalmann and Santelia, 2017). Also in some drought tolerant plants, the accumulation of osmolytes and protective metabolites (such as soluble sugars, proline, trehalose) as well as the induction of ROS scavenging enzymes can partly compromise the down regulation of photosynthetic activity, resulting in considerably (though at reduced rate) sustained growth (Saibo et al., 2009; Hu and Xiong, 2014). This phenomenon is in consistent with the argument that water deficit may even prompt carbon accumulation in certain plants or plant organs (Muller et al., 2011). To this end, plants may engage divergent strategies of water deficit tolerance that could partly reflect their capabilities of acclimating to the fluctuations of water availability status.

Numerous studies have been focused on model plants such as Arabidopsis and rice, and other plant species with which, water deficit occurs occasionally but not persistent over the period of plant development. As the result, drought response mechanisms or tolerant strategies revealed from these plants may be, in certain cases, not applicable to plants suffering the life-time and/or long historic threaten of water deficit. Studies with such persistently water deficit stressed plants seem yet insufficient. In this report, we use an ancient desert shrub, *Ammopiptanthus mongolicus* (Maxim) Cheng f. (Xu et al., 2002; Liu et al., 2013; Wu et al., 2014), to address physiological acclimation to prolonged water deficit stress and to identify early stage response networks that may partly inherit from long historic adaptation to persistent drought stress.

2. Materials and methods

2.1. Plant growth and sample preparation

A. mongolicus seeds were surface sterilized with 70% ethanol, rinsed with distilled water and allowed to soak in water for 2 days before being placed between sheets of moist filter papers for germination at 26 °C. Seedlings were transferred to half strength of Hoagland's solution and grown in a greenhouse at 26 °C with a photoperiod of 16 h light and 8 h dark for 4 weeks. Then the uniform seedlings were treated with half strength of Hoagland's solution with the supplement of 18% (w/v) PEG-6000 to simulate a condition of severe water deficit (equivalents to approx. −0.4 MPa of water potential). Shoot and root samples were collected separately at desired time points. For biochemical measurements and related gene expression analysis, seedlings were sampled at 0, 6, 24, 48 and 72 h after treatments. A proportion of shoot or root samples were collected after 6 h of PEG treatments and used for RNA-Seq analysis. Samples collected at 0 h were taken as control.

2.2. Stomatal assays

Epidermal strips were peeled from 4-week old *A. mongolicus* leaves or rosette leaves of *A. thaliana* and maintained in the experimental solutions under light at 22°Cfor 2 h before microscopic observation. The stomatal opening solution (Low-K) contained: 10 mM KCl, 10 mM MES, pH 6.2 with NaOH and the osmolarity was adjusted to 50 mM by adding sorbitol. The 'High-K' solution contained 50 mM KCl, buffered with 10 mM MES, pH 6.2 and the osmolarity adjusted to 100 mM with sorbitol. For ABA treatments, epidermal peels were submerged into 'Low-K' solution containing 5 μM ABA and incubated for an additional 2 h. Images of stomata were captured from at least 5 visual fields with a numeric light microscope (Nikon, Ti-S). Forty stomata were measured in double blinded manners by Image J software (National Institutes of Health) per treatment from 3 independent experiments. The stomatal aperture was defined as the width to length ratio of a stoma.