The Interaction of the *Arabidopsis* Response Regulator ARR18 with bZIP63 Mediates the Regulation of *PROLINE DEHYDROGENASE* Expression

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ABSTRACT **As the first and rate-limiting enzyme of proline degradation, *PROLINE DEHYDROGENASE1* (*PDH1*) is tightly regulated during plant stress responses, including induction under hypoosmolarity and repression under water deficit. The plant receptor histidine kinases AHKs, elements of the two-component system (TCS) in *Arabidopsis thaliana*, are proposed to function in water stress responses by regulating different stress-responsive genes. However, little information is available concerning AHK phosphorelay-mediated downstream signaling. Here we show that the *Arabidopsis* type-B response regulator 18 (ARR18) functions as a positive osmotic stress response regulator in *Arabidopsis* seeds and affects the activity of the *PDH1* promoter, known to be controlled by C–group bZIP transcription factors. Moreover, direct physical interaction of ARR18 with bZIP63 was identified and shown to be dependent on phosphorylation of the conserved aspartate residue in the ARR18 receiver domain. We further show that bZIP63 itself functions as a negative regulator of seed germination upon osmotic stress. Using reporter gene assays in protoplasts, we demonstrated that ARR18 interaction negatively interferes with the transcriptional activity of bZIP63 on the *PDH1* promoter. Our findings provide new insight into the function of ARR18 and bZIP63 as antagonistic regulators of gene expression in *Arabidopsis*.**

Key words: two-component system; osmotic stress; bZIP63; ARR18; protein interaction; transcription activation; *Arabidopsis thaliana*.

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INTRODUCTION

Stress-related changes in the metabolism of various amino acids are important factors in the adaptation of plants to critical environmental conditions. Proline (Pro) biosynthesis and degradation are especially known to undergo alteration in response to diverse abiotic and biotic stresses. Pro accumulation is a widespread physiological response to water deficit, which is one of the greatest challenges to plant growth and productivity. This accumulation is reversible and the Pro content can decrease back to the initial level upon stress release. Being a small water-soluble zwitterionic molecule, Pro plays a role in osmotic adjustment and protein and membrane stability (Szabados and Savoure, 2010) and may, thus, improve plant tolerance to water stress. In addition to its osmoprotecting (osmolyte) features Pro has become increasingly recognized as a nitrogen and carbon source during after-stress recovery, a

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scavenger of free radicals and a critical amino acid controlling the redox state (Ahmad and Hellebust, 1988; Smirnoff and Cumbes, 1989; Walton et al., 1991; Alia and Saradhi, 1993; Delauney and Verma, 1993; Hare and Cress, 1997). Pro accumulation upon water deprivation is observed in numerous plants, and is achieved through activation of Pro biosynthesis and inactivation of Pro degradation (Yoshiba et al., 1997). The rate-limiting step of Pro biosynthesis in plants is catalyzed by delta1-pyrroline-5-carboxylate synthetase (P5CS), which, in *Arabidopsis thaliana*, is encoded by two genes, *P5CS1* and *P5CS2* (Strizhov et al., 1997). Proline dehydrogenase (PDH), which is also encoded by two genes in *Arabidopsis* (Funck et al., 2010), catalyzes the first and rate-limiting step of Pro degradation (Delauney and Verma, 1993; Peng et al., 1996). Although a lowering of plant water potential correlates with the induction of *P5CS* and the repression of *PDH* genes (Yoshiba et al., 1997), the mechanism of their regulation in response to water stress is less clear. The requirement of abscisic acid (ABA) accumulation for low water potential-induced Pro accumulation (Verslues and Bray, 2006) is in accordance with an, at least partial, dependency of *P5CS* up-regulation on ABA biosynthesis (Sharma and Verslues, 2010). In contrast, the responses of *PDH* to both low water potential and stress release were not impaired in the ABA deficient mutant *aba2-1* (Sharma and Verslues, 2010).

Although *PDH1* expression undergoes extensive regulation by exogenous and endogenous signals, the mechanism of its transcriptional control is still unclear. The accumulation of *PDH1* transcripts, due to hypoosmolarity or supplementation with exogenous Pro, has been shown to be controlled by the ACTCAT *cis*-acting element (ACT-box) in the *PDH1* promoter (Satoh et al., 2002) via bZIP (basic region leucine zipper) transcriptional activators from the S1-group (Satoh et al., 2004).

Detailed analysis of *PDH1* regulation revealed that the S1-group members, bZIP1 and bZIP53, can directly bind to the *PDH1* promoter *in vivo* (Weltmeier et al., 2006; Dietrich et al., 2011). However, promoter activity can be manifoldly enhanced by synergistic co-activation through the heterodimerization of S1-group with C–group bZIPs (Weltmeier et al., 2006). The involvement of S1-group bZIP factors in metabolic reprogramming, due to the transcriptional activation of certain enzymes, including PDH1 and PDH2 (Hanson et al., 2008; Dietrich et al., 2011), is considered to be dependent on cellular energy status, since these bZIPs are tightly regulated by sugars at the translational level (Wiese et al., 2004). Less is known about the functioning of C–group bZIP factors, although the transcriptional regulation of *bZIP63* by ABA (Matiolli et al., 2011) might be indicative of its possible role in water stress response.

Water deficit triggers a number of physiological and biochemical responses, including alterations in hormone levels. ABA and phosphorylation-dependent activation of downstream bZIP transcription factors, predominantly from the A-group, are considered to be key players in water stress responses (Hirayama and Shinozaki, 2007; Wilkinson and Davies, 2010). The precise control of water stress responses also includes ABA-independent processes. Several reports show that the level of cytokinin (CK), which negatively regulates many ABA-mediated physiological processes, is reduced in stressed plants (Alvarez et al., 2008; Argueso et al., 2009). Other factors, including pH, ethylene, gibberellins, sugars, and reactive oxygen species, have also been shown to be altered upon osmotic stress and could be implicated in plant signaling and/or adaptation to changing environmental cues (Alvarez et al., 2008; Schachtman and Goodger, 2008; Wang et al., 2008). Comparatively little, however, is known about the initial steps in water stress responses, including the perception and signaling of water stress. Recent studies indicate that the two-component signaling system (TCS) may be involved in the early steps of low water potential signaling and adaptation to dehydration. Members of three distinct protein classes constitute the plant TCS, creating a canonical multi-step phosphorelay, namely the transfer of a phosphate moiety from conserved histidine to aspartate. These three classes, encoded in *Arabidopsis* by approximately 40 genes, include sensor histidine kinases (AHKs), histidine phosphotransfer proteins (AHPs), and response regulators (ARRs) (Hwang and Sheen, 2001; Lohrmann and Harter, 2002; Grefen and Harter, 2004).

Among the 11 AHKs and HK-like proteins, AHK2, AHK3, and AHK4/CRE1 have been shown to function as CK receptors, with the histidine-to-aspartate phosphorelay being reconstituted in the perception and transduction of the CK signal (Hwang and Sheen, 2001; Lohrmann and Harter, 2002; Hejatko et al., 2009; Muller, 2011). The ethylene (ET) receptor group consists of five genes, namely ETR1, ETR2, ERS1, ERS2, and EIN4. The phosphorelay does not play a major role in ET signaling, as distinct from CK signaling (Voet-van-Vormizeele and Groth, 2008; Hall et al., 2012). AHKs have also been shown to be involved in other processes, such as female gametophyte development (Deng et al., 2010) and the modulation of ABA responses and responses to oxidative, salt, and drought stress (Tran et al., 2007; Desikan et al., 2008; Wohlbach et al., 2008; Tran et al., 2010; Pham et al., 2012; Kumar et al., 2013).

It has been shown that the *Arabidopsis* histidine kinase AHK1 suppresses lethality in the yeast *sln1Δsho1Δ* double mutant, lacking its endogenous osmosensors, under high salinity stress and activates the *HIGH OSMOLARITY GLYCEROL RESPONSE1* (HOG1) mitogen-activated protein kinase pathway (Urao et al., 1999). The osmosensor function of AHK1 in yeast requires histidine kinase activity (Urao et al., 1999). Likewise, AHK1 has been shown to act as a positive regulator of drought and salt stress responses in *Arabidopsis*, functioning upstream of both ABA-mediated and ABA-independent signaling pathways, and was thus suggested to directly act as a plant osmosensor (Tran