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Over-expression of *GsZFP1*, an ABA-responsive C2H2-type zinc finger protein lacking a QALGGH motif, reduces ABA sensitivity and decreases stomata size

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ABSTRACT

A cDNA of the gene *GsZFP1* was cloned from *Glycine soja*. *GsZFP1* encodes a protein with one C2H2-type zinc finger motif. The QALGGH motif, which exists in most plant C2H2-type zinc finger proteins (ZFPs), does not exist in *GsZFP1*. Real-time RT-PCR revealed that *GsZFP1* expression was significantly up-regulated by exogenous ABA, both in leaves and roots. Over-expression of this gene, in *Arabidopsis thaliana*, resulted in a reduced sensitivity to ABA during seed germination and seedling growth. Transcript levels of some stress and ABA marker genes, including *RD29A*, *RD22*, *NCED3*, *COR47*, *COR15A* and *KIN1* were increased, in the *GsZFP1* over-expression lines, when plants were treated with exogenous ABA. We further studied the effects of *GsZFP1* over-expression on the regulation of genes involved in ABA signaling. Negative ABA signaling regulators, such as *ABI1* and *ABI2*, were up-regulated in over-expression lines, while positive ABA signaling regulators, such as *ABF4*, *ABI5*, *GTG1*, *GTG2*, *PYR1/RCAR11*, *PYL2/RCAR13*, *SnRK2.2* and *SnRK2.3*, were down-regulated, in comparison to wild type plants. *GsZFP1* over-expression lines also exhibited small stomata, impairment of ABA-induced stomata closure. The data presented, herein, suggests that *GsZFP1* plays a crucial role in ABA signaling in *A. thaliana*, *GsZFP1* may be a promising gene for negative regulating ABA signaling. Our findings broaden our understanding of this C2H2 ZFP subtype's function, and add to the body of evidence that has been developed in earlier studies.

Introduction

The hormone abscisic acid (ABA) regulates plant development and stress responses (Cutler et al., 2010), predominantly through the regulation of downstream gene expression. The application of ABA to Arabidopsis seedlings leads to the differential expression of more than 1300 genes, some of which code for proteins involved in signal transduction (Hoth et al., 2002). In recent years, several key regulators of ABA signal transduction have been identified (Himmelbach et al., 2003), including membrane-associated proteins, phospholipases C/D, G proteins, type-2C/A protein phosphatases (PP2C/A), and various protein kinases, which include SNF1-related protein kinases (SnRKs) (Hirayama and Shinozaki, 2007). ABA receptors are the most upstream components in ABA signaling, and ABA signal perception by ABA receptors is considered to be the primary event that triggers downstream signaling cascades to induce the final physiological responses, and research in this field has attracted much attention, several different ABA receptors have also been identified, where perception is performed by plasma membrane proteins and intracellular proteins (Finkelstein et al., 2002; Verslues and Zhu, 2007). Recently, the plant family of PYR/PYL/RCAR was identified as a reliable family of ABA receptors, and ABA recognition by the PYR/PYL/RCAR family of proteins activates the SnRK2 family of protein kinases through inactivation of their central negative regulators, the type 2C protein phosphatases (PP2Cs) (Cutler et al., 2010; Ma et al., 2009; Park et al., 2009; Santiago et al., 2009). These receptors regulate ABA responses by controlling SnRK2 kinase activity and the phosphorylation of downstream target proteins such as ABFs, which control nuclear responses (Kobayashi et al., 2005), and the ion channel SLAC1,

Abbreviations: ABA, abscisic acid; C2H2, Cys2/His2; *G. soja*, *Glycine soja*; qRT-PCR, quantitative real-time PCR; RT-PCR, reverse transcription PCR; WT, wide type; ZFPs, zinc finger proteins.

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which is thought to be a slow-type anion channel (Vahisalu et al., 2008), was shown to undergo phosphorylation via an SnRK2 family protein kinase and induce depolarization of the plasma membrane (Geiger et al., 2009). In addition to PYR/PYL/RCAR family proteins, several candidate ABA receptors have been reported, a novel class of G protein-coupled receptor (GTG1 and GTG2), which have been reported to interact with GPA1 to regulate ABA signaling have been reported (Pandey et al., 2009); there is also CHLH/ABAR, which functions as a receptor for ABA, interacting with a group of WRKY transcription factors which directly regulate a set of ABA responsive transcription factors, such as ABI4, ABI5, ABF4, and MYB2, that, in turn, regulate many key genes involved in ABA-induced physiological responses (Shang et al., 2010; Shen et al., 2006), the molecular explanation for how Arabidopsis CHLH regulates the myriad ABA-controlled processes will be required to fully comprehend the role of this protein in ABA signaling, although this model faces criticisms since two groups have failed to show ABA binding by barley or Arabidopsis CHLH (Müller and Hansson, 2009; Tsuzuki et al., 2011).

Previous studies have shown that the transcriptional factors play important roles in plant mediating ABA responses. These complex transcriptional regulation processes are mainly achieved by transcription factors having the ability of activating or repressing the expression of the related genes (Chen and Zhu, 2004), for example, many WRKY transcription factors have been described involved in ABA signaling (Ren et al., 2010; Shang et al., 2010), all the WRKY proteins contain one or two conserved WRKY domains which are about 60 amino acids and have the DNA binding activity specific to the DNA sequence (C/T) TGAC (T/C) (named as W-box) (Ulker and Somssich, 2004). The C2H2-type zinc-finger proteins (ZFPs), also called the TFIIIA-type zinc-finger proteins, represent a large family of eukaryotic transcription factors. The zinc finger domain is one of the most important structural motifs involved in protein–DNA interactions, and is also known to be involved in binding of proteins in plants. The classical zinc-finger is a small domain consisting of ~30 amino acids and the amino acid consensus sequence is $CX_{2-4}CX_3FX_5LX_2HX_{3-5}H$. In Arabidopsis, a total of 176 proteins that contain one or more zinc finger domains have been reported, thus making ZFPs to be one of the largest families of putative transcriptional regulators (Englbrecht et al., 2004). Several plant members of this family have various regulatory roles in ABA signaling (Drechsel et al., 2010; Li et al., 2010); ZFPs of the C2H2-subtype typically lack transactivation activity, and instead act as repressors of gene expression, blocking the transactivation ability of other transcription factors (Drechsel et al., 2010; Ohta et al., 2001). C2H2-type ZFPs typically contain a QALGGH motif within the ZFP domain. In recent years, several plants C2H2-type ZFPs, without the QALGGH motif, have been identified by sequence-independent genetic studies, examples of which include *AtFIS2*, *AtTT1*, *ZmID1* and *StPCP1* (Sagasser et al., 2002).

There are few reports investigating the role of QALGGH-deficient C2H2-type ZFPs in ABA signal transduction, with an exception being the case of *GhDi19-1* and *GhDi19-2*. Over-expressing *GhDi19-1* or *GhDi19-2* in Arabidopsis led to an increased sensitivity to salinity and exogenous ABA (Li et al., 2010). The roles of C2H2-type ZFPs, especially QALGGH-deficient sub-types, in ABA signaling pathways thus remain to be explored in greater detail.

Here we report the identification of a novel *Glycine soja* (*G. soja*) C2H2-type ZFP, named *GsZFP1*, whose function in ABA signaling we sought to elucidate. We generated transgenic Arabidopsis that ectopically expressed *GsZFP1*, and identified three homozygous lines for further study. We then examined the transcript levels of ABA-response genes, and concluded with an examination of the effects of *GsZFP1* over-expression on stomata development and ABA-induced stomata closure.

Materials and methods

Plant materials, growth conditions and stress treatments

Seeds of *Glycine soja* G07256 were obtained from the Jilin Academy of Agricultural Sciences (Changchun, China). For *GsZFP1* gene expression analysis under ABA treatment, Nineteen days seedlings of *G. soja* were transferred into 0.5× MS solution, cultured two days, and then transferred into 0.5× MS solution with without 100 μM ± ABA. Equal amounts of leaves and roots were sampled at 0.5 h, 1 h, 3 h, and 6 h time points after treatment, and equal amounts of leaves and roots were sampled at 0 h, 0.5 h, 1 h, 3 h, and 6 h time points, respectively, under control conditions.

Wild-type (WT) *Arabidopsis thaliana* (Columbia ecotype; from Northeast Agricultural University, Harbin, China) were used for transformation. For the expression analysis of ABA responsive genes, seeds from WT and *GsZFP1* over-expression lines were sown on filter paper saturated with 0.5× MS solution. After 21 days of growth, seedlings were incubated at 0.5× MS solution with 100 μM ABA. Samples were taken from three biological replicates at 1 h, 3 h, 6 h and 12 h time after treatment and, equal amounts of samples were sampled at 0 h, 1 h, 3 h, 6 h and 12 h time points, respectively, under control conditions.

ABA was ordered from Sigma (±abscisic acid, A1049-250MG, Plant Cell Culture Tested. ~99% HPLC), it was dissolved in ethanol anhydrous (ET0737, Purity > 99.5%, Water < 0.3%, Sangon of Shanghai city, China) 100 μM/ml at first, and then diluted into different concentrations with ddH_2O, and pH was 5.8 which was the same as 0.5× MS solution. For *GsZFP1* and ABA responsive genes expression analysis under 100 μM ABA treatment, germination experiment under 0.8 μM ABA treatment, root length experiment under 30 and 40 μM ABA treatment, stomata closure experiment under 10 μM ABA treatment, due to the ABA was dissolved in ethanol anhydrous, the final alcohol concentration (volume/volume) of the 0.5× MS solution is: 1/1000, 0.008/1000, 0.3/1000 and 0.4/1000, 0.1/1000, respectively; so in each one liter 0.5× MS solution which was used under control conditions was supplemented with 1 ml, 8 μl, 0.3 ml or 0.4 ml, 0.1 ml ethanol anhydrous, respectively, so as to make the final alcohol concentration was the same with the 0.5× MS solution which supplemented ABA solution.

Isolation and sequence analysis of GsZFP1

The full-length cDNA of *GsZFP1* was retrieved by homologous cloning. Briefly, total RNA was isolated from whole seedlings of *G. soja* G07256 using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), and the cDNA was prepared using the SuperScript™ III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA). The full-length cDNA of *GsZFP1* was obtained using primers based on the *Glycine max* homologue (primers: 5′- ATGGCCTTAAATTCTCCAAACT-3 and 5′-CTAGCTAG-CTACAGGCTCCAATTT-3′). The PCR product was cloned into the pGEM-T cloning vector (Promega, Madison, WI) and subjected to sequencing. Sequence analysis and alignments were performed with Blastp and ClustalX. A phylogenetic tree was generated using the neighbor-joining method with the MEGA4.0 software.

Quantitative real-time PCR

Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA); the isolated RNA was subjected to reverse transcription using the SuperScript™ III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA) with the oligo dT_{18} reverse primer. Prior to the qRT-PCR assays, the quality of the cDNA was assessed by PCR using GADPH-specific primers to test for genomic DNA contamination. One microliter of synthesized cDNA (diluted 1:5) was