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RESEARCH ARTICLE

A Stress-Responsive CaM-Binding Transcription Factor, bZIP4, Confers Abiotic Stress Resistance in Arabidopsis

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Abstract

The basic leucine zipper (bZIP) transcription factors (TFs) function as regulators of many key developmental and physiological processes in all eukaryotes. In this study, we characterized the function of Arabidopsis bZIP4, a group S bZIP, whose function was not known. We confirmed that bZIP4 localizes to the nucleus and has DNA-binding affinity. By qRT-PCR and GUS histochemical analysis, we showed that *bZIP4* is specifically expressed in root and that its expression is induced by abiotic stress and ABA. By phenotypic analysis, we demonstrated that the root length and the germination rate of *bZIP4* overexpression (*bZIP4*-Ox) were significantly longer and higher than those of the WT and *bZIP4*-SRDX under higher salt and glucose concentrations, indicating that *bZIP4*-Ox is insensitive and tolerant to abiotic stress. Despite that, we found that *bZIP4*-Ox had enhanced expression of genes encoding protein phosphatases suppressing ABA responsiveness. We also confirmed that bZIP4 interacts with CaM1 and showed that its DNA-binding affinity is inhibited by interaction with CaM1. We propose a model in which the increased cytosolic calcium concentration under stress conditions activates CaM1 to bind bZIP4 to remove it from promoters of genes encoding ABA negative regulators, allowing the plants to operate on a typical ABA signaling pathway.

Keywords ABA · Abiotic stress · *Arabidopsis thaliana* · BZIP4 · CaM1

Abbreviations

ABA	Abscisic acid
BiFC	Bimolecular fluorescence complementation
bZIP4	Basic region leucine zipper protein 4
CaM1	Calmodulin1
CAMTA	CaM-binding transcription activator
GFP	Green fluorescence protein
GST	Glutathione S-transferase
GUS	Beta-glucuronidase
His	Poly-histidine
K.O	Knock-out
MS medium	Murashige and skoog medium
Ox	Overexpression
qRT-PCR	Quantitative real-time reverse transcription-polymerase chain reaction
RT-PCR	Reverse transcription-PCR
TF	Transcription factor
WT	Wild type
X-gluc	5-Bromo-4-chloro-3-indolyl glucuronide
YFP	Yellow fluorescence protein

Introduction

Abscisic acid (ABA) is a well-studied phytohormone that is known to function in various plant developmental processes, such as seed dormancy and development (Finkelstein et al. 2002), primary root growth and lateral root branching (Sun et al. 2018), and plant senescence (Gao et al. 2016), as well as in response to various abiotic stresses, such as drought (Fujita et al. 2011), salinity (Zhang et al. 2006), heat and cold (Zhang et al. 2019). Signaling pathway cascades mediated by ABA help plants to adapt to the external conditions that keep changing and sometimes harmful to the plant (Umezawa et al. 2010).

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Most ABA-responsive genes contain consensus *cis*-elements, ABRE (ABA-responsive element), in their promoter regions. The ABRE-binding factor (AREB/ABF) family proteins are transcription factors (TFs) that bind to these *cis*-elements and regulate ABA responses. These AREB/ABF family proteins belong to the group A cluster of the basic leucine zipper protein (bZIP) family. Group A consists of 13 members of bZIP proteins, whose expression is induced in response to different stresses, despite of having partial redundancy (Banerjee & Roychoudhury 2017). In Arabidopsis, a total of 78 bZIP proteins have been identified and classified into 13 different groups; A ~ K, M and S (Droge-Laser and Weiste 2018). These bZIPs are involved in signaling pathways, such as hormone response, stress response, and plant development (Weiste and Droge-Laser 2014; Hartmann et al. 2015; Weiste et al. 2017). Of them, group S is the largest cluster of the bZIP family (Ehlert et al. 2006; Jakoby et al. 2002) and it can be divided into S_1, S_2 and S_3 (Ehlert et al. 2006). Of these three subgroups, only subgroup S_1 members have been well studied and known to form a heterodimer with group C bZIP family proteins to build a C/S_1-bZIP network, regulating energy homeostasis in plant (Droge-Laser et al. 2018). Moreover, it is also known that some bZIPs in subgroup S_1 regulate development and stress responses in roots (Hartmann et al. 2015; Weiste et al. 2017).

Calcium ions (Ca^{2+}) are present in the cell substrate of not only plants but also all living organisms. Ca^{2+} works as a second messenger that plays an important role in biological processes in cells. Organisms employ a set of proteins for Ca^{2+} influx as well as efflux. The combination of Ca^{2+} influx through Ca^{2+} channels and active Ca^{2+} efflux through energy-dependent Ca^{2+} transporters, produces temporal and spatial changes of Ca^{2+} concentration in the cytoplasm or organelles (Clapham 2007; Kudla et al. 2010). Moreover, when plants face stressful stimuli caused by abiotic conditions, especially by drought or salinity, ABA and cytosolic-free Ca^{2+} control, the activity of transport proteins involved in rapid signaling events to help the plants to respond to the stress (Levchenko et al. 2005; Huang et al. 2019). The signaling pathways of Ca^{2+} and ABA are not clearly independent from each other, and integration occurs in some stages (Maierhofer et al. 2014; Deger et al. 2015), but the way they affect each other remains still elusive. The importance of Ca^{2+}-binding proteins in plants is reflected by their large number and diversity; for example, 250 proteins carry one or more EF-hand Ca^{2+}-binding motifs in Arabidopsis. Calmodulin (CaM), a typical Ca^{2+} sensor, is a small, highly conserved protein in all eukaryotic cells. The protein has two approximately symmetrical globular domains each containing a pair of EF-hand motifs, for a total of four Ca^{2+}-binding sites. CaM mediates many crucial processes including plant development and adaptation to environmental stimuli (Snedden and Fromm 1998). Numerous CaM-binding proteins have been reported. Some of them called CaM-binding transcription activators (CAMTAs) are TFs, such as MYB, WRKY and bZIP, binding with a CaM (Doherty et al. 2009). These CaMTA are presumed to play a role in stress response, but the detailed signaling networks have not been fully studied.

The aim of this study was to characterize the function of Arabidopsis bZIP4, one member of group S bZIP, whose interaction with bZIP1 has been reported, but whose function is unknown (Ehlert et al. 2006). We performed subcellular localization to confirm the nuclear localization of bZIP4 as a TF and then performed an EMSA to determine whether bZIP4 has its DNA-binding affinity with C-box and Hex-motif sequences as do bZIP proteins. We then analyzed *bZIP4* expression by qRT-PCR to identify its tissue-specific expression pattern and to characterize whether its expression is induced by abiotic stress and ABA. We also constructed and selected each two homozygous lines of *bZIP4*-Ox and *bZIP4*-SRDX and compared their phenotypes with the WT under normal and stress conditions. In addition, we provided evidence that bZIP4 interacts with CaM1, a typical Ca^{2+} sensor protein, by in vitro pull-down assay and in vivo BiFC assay, and that their interaction inhibits the ability of bZIP4 to bind to promoters of negative regulators of ABA in a higher Ca^{2+} ion conditions, demonstrating that overexpression of bZIP4, a root-specific TF, confers abiotic stress resistance.

Results and Discussion

bZIP4 is a Root-Specific TF and Responsive to Abiotic Stress

Arabidopsis *bZIP4* is known to belong to group S, which is the largest bZIP group in Arabidopsis. bZIP4 also does not have any conserved domains other than the bZIP domain. Arabidopsis group S bZIPs are further sub-grouped into S_1, S_2 and S_3 based on their sequence homology (Ehlert et al. 2006). To identify the subgroup of bZIP4, we aligned the deduced amino acid sequences of Arabidopsis group S bZIPs (Supplementary Fig. S1) and confirmed that bZIP4 belongs to group S_3, consistent with a previous report (Ehlert et al. 2006). As expected, the bZIP domain was solely conserved and the rest of the sequences in the N-and C-terminal regions greatly varied among the group members. These variable sequences contribute to divide the group into subgroups and to confer each protein group a differentiated function.

To confirm the nuclear localization of bZIP4 as a TF, we made a smGFP-tagged bZIP4 construct (*Pro35S:bZIP4-smGFP*) and observed its subcellular localization in onion epidermal cells. We found that the smGFP fluorescence

emitted by bZIP4-smGFP was detected in the nucleus, overlapping the fluorescence signal from DAPI staining (Fig. 1b), while the fluorescence was detected throughout the cytosol when only smGFP (*Pro35S:smGFP*) was expressed (Fig. 1a). Then, to determine whether bZIP4 has DNA-binding affinity, we performed an EMSA of bZIP4 with probes of C-box (TGCTGACGTA) (Song et al. 2008) and Hex-motif (CTGACGTGGC) (Kang et al. 2010) elements (Supplementary Table S1), which contain ACGT-based consensus sequence motifs known to be bound by bZIP family proteins (Izawa et al. 1993). As a result, we found that the band intensity of bZIP4 for both probes increased in proportion to the concentration of the protein, indicating that bZIP4 has DNA-binding activity and binds to the consensus sequence for bZIP family proteins (Fig. 1c). Because bZIP1 is a positive regulator of plant tolerance to abiotic stress (Kang et al. 2010), and because bZIP4 is the only subgroup S_3 protein that interacts with bZIP1, although weakly (Ehlert et al. 2006), we examined whether bZIP4 also functions in response to abiotic stress. We first analyzed *bZIP4* expression patterns using qRT-PCR after treating plants with ABA, drought and cold. We observed that the transcript level rapidly increased and then gradually decreased within 1–3 h under ABA treatment, whereas the expression was induced within 1 h or 9 h by drought or cold stress treatment, respectively, and gradually increased until 9 h or 12 h, respectively. This result demonstrates that *bZIP4* is highly responsive to abiotic stress (Fig. 2a), like other *bZIP* genes in group S, such as *bZIP1*, *bZIP11* and *bZIP53* (Hartmann et al. 2015; Weiste et al. 2017).

Then, we examined *bZIP4* gene expression patterns in six different tissues of Arabidopsis; rosette leaf, cauline leaf, stem, flower, seedling, and root, by qRT-PCR, and found that its expression was at basal level in all tissues, but was very specifically and strongly detected in roots as compared to other tissues (Fig. 2b). We then constructed two transgenic lines of *ProbZIP4:GUS* each harboring either the 2,245-bp or 2,071-bp promoter regions upstream from the start codon of *bZIP4*, and found that $ProbZIP4_{2,245}$*:GUS* produced much stronger GUS expression than $ProbZIP4_{2,071}$*:GUS* in roots (Supplementary Fig. S2). Thus, we examined the intensive tissue-specific GUS expression in the $ProbZIP4_{2,245}$*:GUS* line and found that GUS was expressed exclusively in roots at all different developmental stages from seedling (Fig. 2c–f) to mature plants (Fig. 2g–i). Roots are the primary sites where plants respond directly to many abiotic stresses, such as salt, drought, and cold. ABA expression in the roots is also induced by these external stresses (Hong et al. 2013), and plants respond to and withstand abiotic stress through ABA signaling pathways (Zhang et al. 2006; Vishwakarma et al. 2017). Thus, we conjectured that bZIP4 also plays a role in abiotic stress response pathway via ABA.

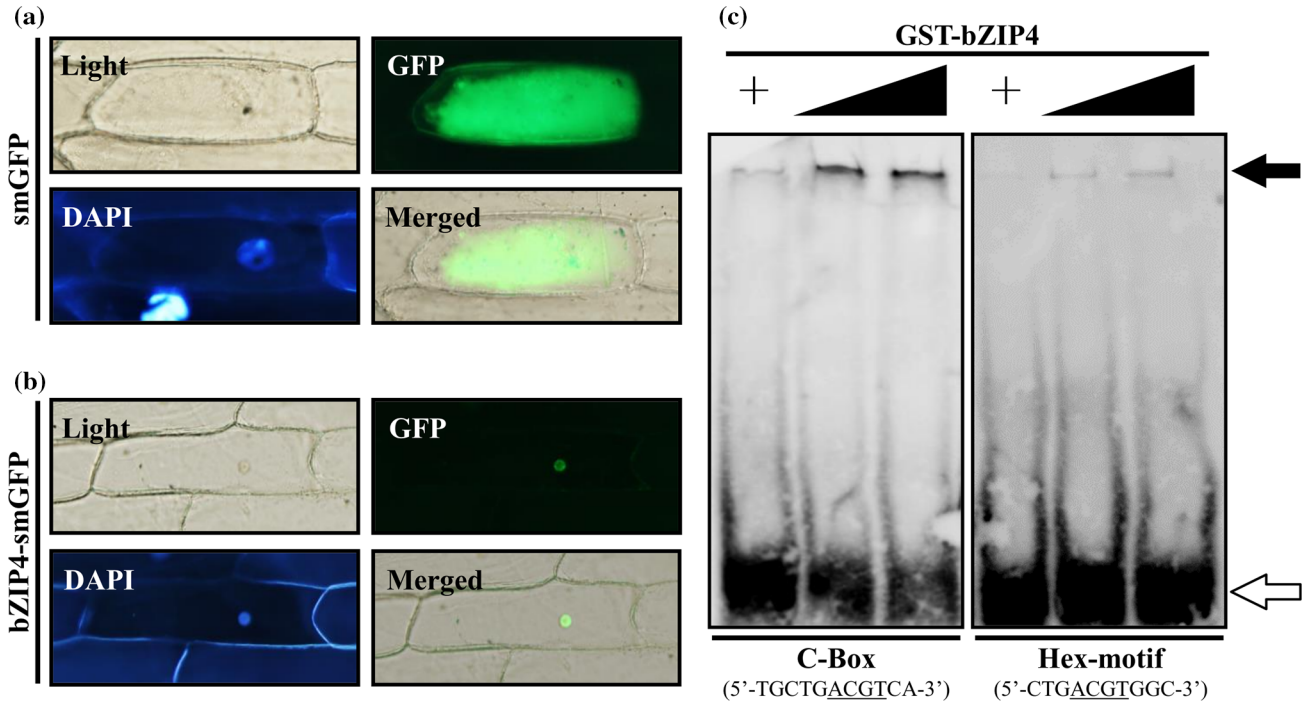

Fig. 1 Subcellular localization and EMSA of bZIP4. Subcellular localization of smGFP (**a**) and bZIP4-smGFP (**b**). *Pro35S:smGFP* and *Pro35S:bZIP4-smGFP* were introduced into onion epidermal cell through particle bombardment and observed by in vivo imaging 1 day after the introduction. DAPI was used as a nucleus marker. (**c**) EMSA of bZIP4 with 3′-biotin-labeled probes using C-box element and Hex-motif element. Black arrow indicates the shifted bands. White arrow indicates free probes

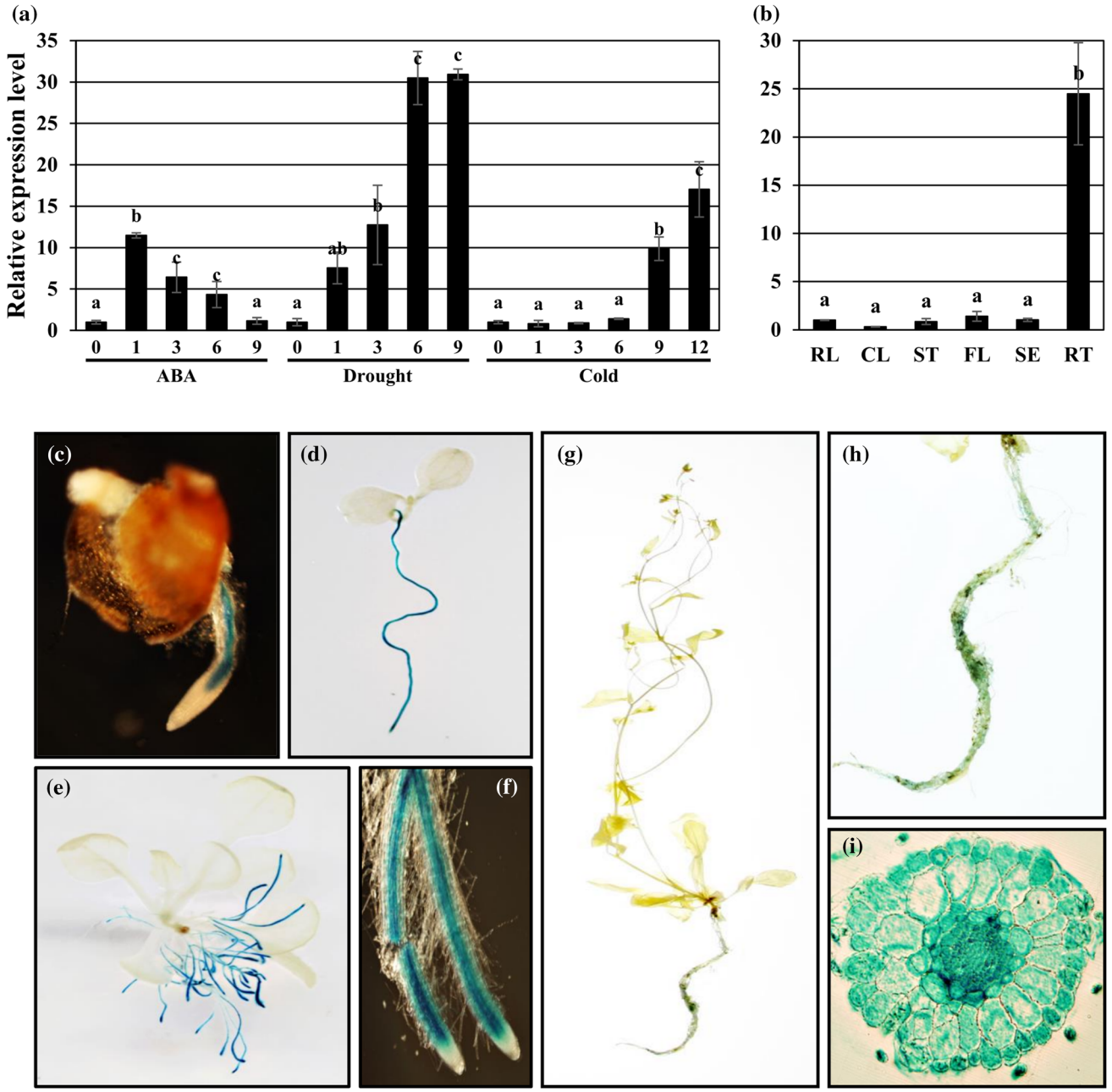

Fig. 2 Quantitative RT-PCR and histochemical GUS analysis of *bZIP4* expression in Arabidopsis. **a** Real-time PCR of *bZIP4* expression under abiotic stress conditions. RNA was extracted from 2-week-old plants grown on MS medium after treating them with abiotic stresses; 100 μM ABA, drought, and cold. The expression level of *eIF4A1* was used as an internal control. The expression level of *bZIP4* in untreated seedlings of the WT was set to 1.0. Data represent the mean ± standard deviation of three individual experiments. Different letters indicate significant differences among times after treatments ($p < 0.05$, one-way ANOVA followed by Tukey's HSD test). **b** Real-time PCR for tissue-specific expression of *bZIP4*. RNA was extracted from 4-week-old plants grown on soil under long-day conditions (16/8, light/dark). *RL*, Rosette leaf; *CL*, cauline leaf; *ST*, stem; *FL*, flower; *SE*, seedling; *RT*, root. The expression level of *eIF4A1* was used as an internal control. The expression level in rosette leaf was set to 1.0. Data represent the mean ± standard deviation of three individual experiments. Different letters indicate significant differences among different tissues ($p < 0.05$, one-way ANOVA followed by Tukey's HSD test) **c–i** Histochemical analysis of GUS expression pattern in *ProbZIP4:GUS*. **c** 2-day-old Seedling, **d** 7-day-old seedling, **e** 17-day-old plant, **f** roots, **g** whole plant, **h** root of **g**, and **i** transverse section of mature plant root

bZIP4 Overexpression Confers Abiotic Stress Resistance but Enhances the Expression of Genes Encoding Protein Phosphatases Suppressing ABA Responsiveness

To investigate the physiological effect of bZIP4 in Arabidopsis, we constructed *bZIP4* overexpression lines driven by the CaMV 35S promoter and selected two T_3 homozygous lines, named Ox 9–4 and Ox 18–2 (Fig. 3a). We also constructed SRDX-dominant repression lines by tagging *bZIP4* with the SRDX motif, driven by CaMV 35S promoter (Fig. 3b) (because a T-DNA insertional knock-out mutant was not available). We selected two independent lines, named SRDX 14–1 and SRDX 15–1 (Fig. 3c). Then, we examined the sensitivity of *bZIP4* transgenic plants to salt stress and glucose-induced germination delay. The WT, *bZIP4*-Ox lines (Ox 9–4, Ox 18–2), and *bZIP4*-SRDX lines (SRDX 14–1, SRDX 15–1) were grown for 2 weeks on 1/2 MS agar media with normal, high salt (50 and 100 mM NaCl) and high sugar [2 and 4% (w/v) glucose] concentrations. With normal growth media, both *bZIP4*-Ox and *bZIP4*-SRDX lines were not morphologically different from the WT. However, high concentrations of salt or glucose significantly shortened the roots of the WT and *bZIP4*-SRDX seedlings, whereas roots of the *bZIP4*-Ox seedlings were significantly longer than those of the WT and *bZIP4*-SRDX, demonstrating that *bZIP4*-Ox

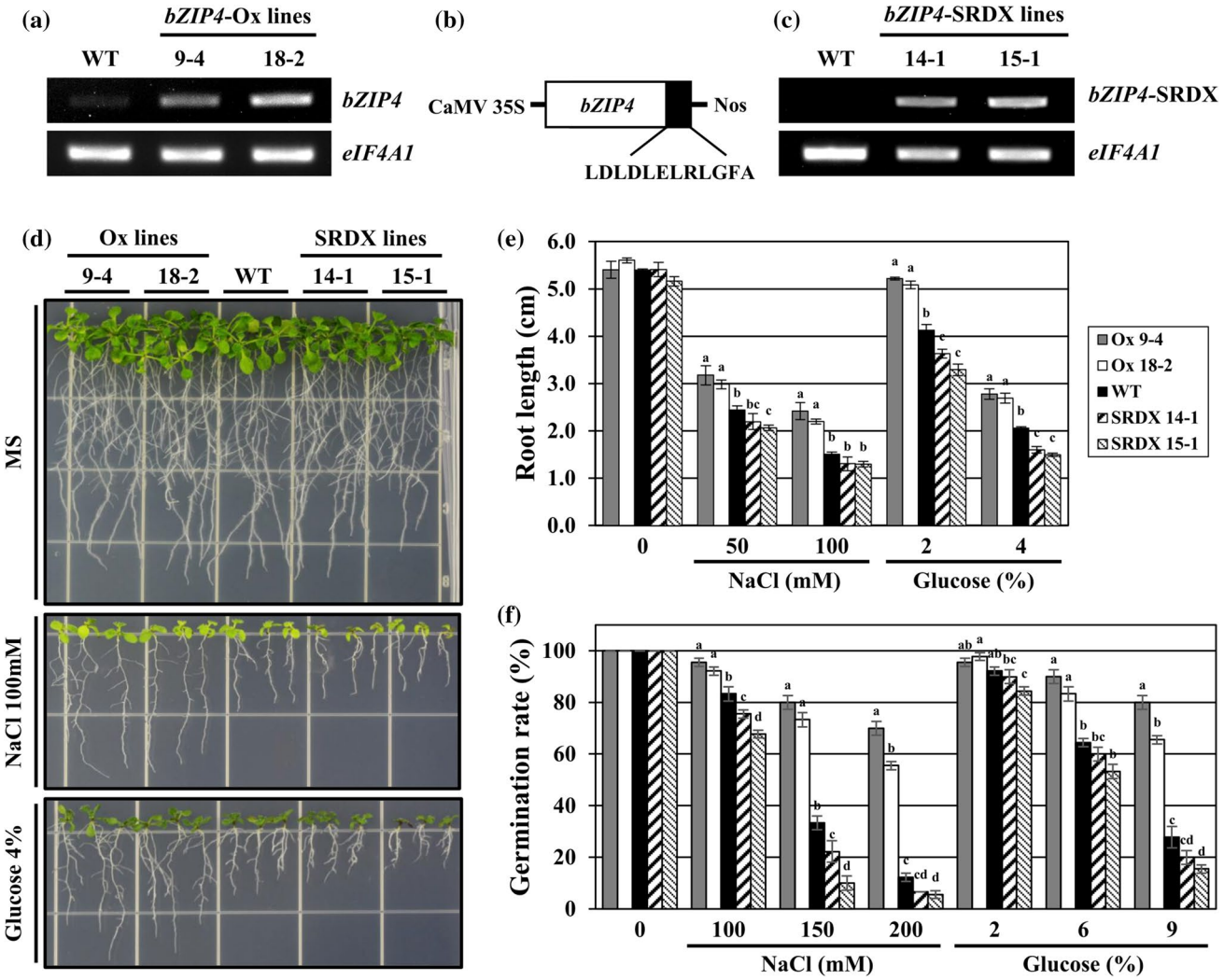

Fig. 3 Confirmation of *bZIP4* overexpression and repression transgenic lines and their phenotypes to abiotic stress. **a** RT-PCR of *bZIP4*-Ox lines. **b** Construct model of *bZIP4*-SRDX target gene repressing system. **c** RT-PCR of *bZIP4*-SRDX lines. **d** Root growth of *bZIP4*-Ox lines, WT, and *bZIP4*-SRDX lines under normal and abiotic stress conditions. **e** Measurement of root length of *bZIP4*-Ox lines, WT, and *bZIP4*-SRDX lines under normal and abiotic stress conditions. **f** Measurement of germination rate of *bZIP4*-Ox lines, WT, and *bZIP4*-SRDX lines under normal and abiotic stress conditions. Data represent the mean ± standard deviation of three individual experiments. Different letters indicate significant differences among transgenic lines at each time point after treatment ($p < 0.05$, one-way ANOVA followed by Tukey's HSD test)

lines are insensitive to salt and glucose (Fig. 3d, e). We also examined the germination rate of the WT and *bZIP4* transgenic lines under high salt and glucose conditions. As a result, *bZIP4*-overexpression lines showed a substantially higher germination rate than the WT despite increasing concentrations of salt and glucose, while those of *bZIP4*-SRDX lines had much reduced germination rate compared to the WT under higher salt and glucose concentrations (Fig. 3f). These results indicate that *bZIP4* overexpression in Arabidopsis confers tolerance against abiotic stress.

Salt stress and sugar response are known to regulate seed dormancy and root length via ABA signaling and biosynthesis (Zhang et al. 2006; Dekkers et al. 2008; Vishwakarma et al. 2017). Moreover, it is known that exogenous high glucose treatment induces ABA accumulation and accelerates ABA response (Arenas-Huertero et al. 2000), and that bZIPs in group S_1 are involved in this sugar signaling pathway (Hartmann et al. 2015; Weiste et al. 2017; Kang et al. 2010). Since we confirmed that the expression of *bZIP4* is affected by ABA (Fig. 2a), we analyzed the expression levels of genes encoding negative regulators of ABA signaling (*ABI1*, *ABI2* and *PP2CA*) (Leung et al. 1997; Rodrigues et al. 2013), ABA biosynthesis (*NCED3*; nine-cis-epoxycarotenoid dioxygenase 3) (Iuchi et al. 2001), and stress-induced proteins (*KIN2*, *RD22*) (Abe et al. 1997; Wang et al. 1995) in the WT, *bZIP4*-SRDX lines, and *bZIP4*-Ox lines after ABA treatment. As a result, the expression of genes *ABI1*, *ABI2* and *PP2CA*, involved in suppressing ABA response, were greatly enhanced in *bZIP4*-overexpression lines, but they were not suppressed in *bZIP4*-SRDX lines (Fig. 4), suggesting that bZIP4 promotes the suppression of ABA response, subsequently reducing the effect of ABA in stress responsiveness. Moreover, *bZIP4* overexpression enhanced the expression of genes involved in resistance to abiotic stress, such as *KIN2*, encoding the stress-induced protein KIN2. In addition, the expression of *NCED3*, the gene encoding a key enzyme in the biosynthesis of ABA, was also increased, but its expression could be increased regardless of the ABA response (Tan et al. 2018). On the other hand, expression of *RD22* (*responsive to desiccation 22*), taking part in the ABA signaling pathway different from the PYR/PYL–PCAR–PP2Cs–SnRK2 pathway in which ABI1, ABI2 and PP2CA are involved, was inconsistent and seemed not to be affected by overexpression of *bZIP4*. Taken together, these results suggest that bZIP4 enhances the expression of genes encoding negative regulators of ABA signaling, but overexpression of *bZIP4* confers abiotic stress resistance.

bZIP4 Interacts with CaM1, the Calcium-Binding Messenger Protein

Calcium ion ($Ca2^{+)}$ plays an important role in plant stress response. Since Ca^{2+} signaling is tightly regulated by abiotic stress responses, such as drought and salinity, it has been suggested that Ca^{2+} signaling could be closely associated with the ABA pathway (Edel and Kudla 2016), but the link between these two pathways has not been fully studied. Since protein interactions between bZIP4 and various CaMs or calmodulin-like proteins (CMLs) have been proposed (Popescu et al. 2007), we conjectured that the interaction between bZIP4 and CaM1 could be a link between the two giant signaling pathways (Ca^{2+} and ABA), positioning them in an integrated network. Therefore, we decided to test whether there is an interaction between these two proteins.

To confirm the interaction between bZIP4 and CaM1, we first performed an in vitro pull-down assay using the purified GST-tagged bZIP4 and His-tagged CaM1 in the presence or absence of Ca^{2+}. As a result, we confirmed that bZIP4 and CaM1 interact in vitro in the presence of Ca^{2+} (Fig. 5a). Then, we carried out the BiFC assay in onion epidermal cells using *Pro35S:bZIP4-YFP*^{N} and *Pro35S:CaM1-YFP*^{C} constructs and verified that bZIP4 and CaM1 interact with each other in vivo (Fig. 5b).

DNA-Binding Affinity of bZIP4 to its Target Promoters is Inhibited by Interaction with CaM1 in the Presence of Calcium Ion

CaM acts as a messenger protein and, when activated by binding to calcium ions, is known to bind to other proteins and regulate a variety of signal transduction pathways (Ranty et al. 2006; Reddy et al. 2011). Since the interaction between bZIP4 and CaM1 was confirmed in vitro and in vivo, we tried to find out whether their interaction could also affect the ABA signaling pathway. Since it has been reported that DNA-binding affinity of TFs can be changed by binding of CaM proteins (Yoo et al. 2005; Zhou et al. 2018), we wondered whether the DNA-binding affinity of bZIP4 could also be altered by CaM1 binding. To investigate the effect of CaM1 binding to bZIP4, we performed EMSA of bZIP4 with a C-box probe in the presence of CaM1. As a result, the DNA-binding affinity of bZIP4 decreased as the concentration of CaM1 increased (Fig. 6), indicating that CaM1 binding to bZIP4 inhibits its binding to the target promoters. Taken altogether, bZIP4 enhances the expression of negative regulators of ABA signaling, and the DNA-binding affinity of bZIP4 to its promoters is inhibited by its binding with CaM1 in higher Ca^{2+} conditions, subsequently inducing tolerance to abiotic stress (Fig. 7). Therefore, we suggest that the interaction between bZIP4 and CaM1 could explain the integration of responses to external signals by the ABA-mediated and calcium-mediated signaling pathways. In this study, we mainly dealt with CaM1 as a CaM protein that interacts with bZIP4, but according to a previous study, several additional CaM and CML proteins could interact with bZIP4 (Popescu et al. 2007). Future research on the

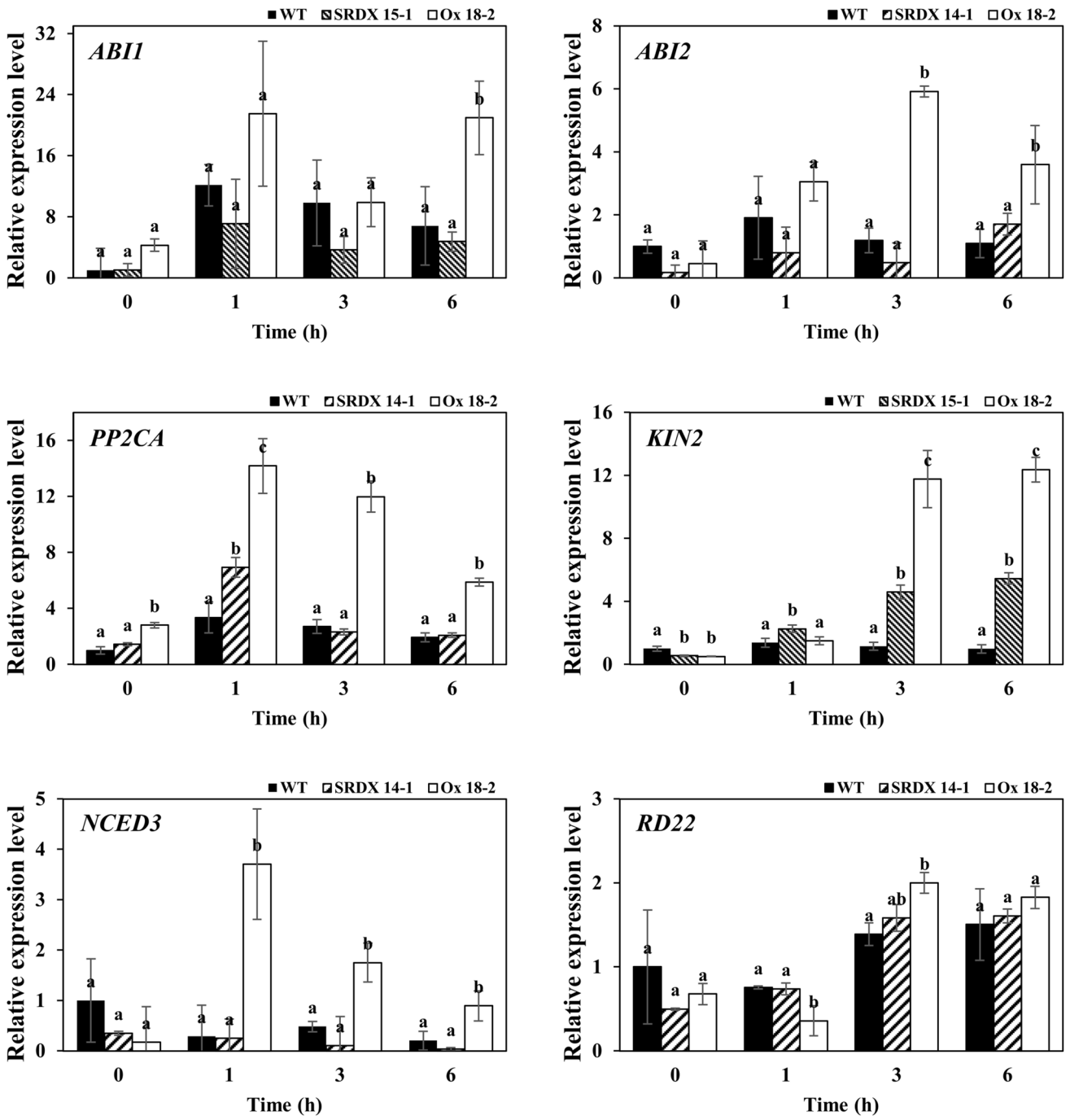

Fig. 4 Expression pattern of genes related to ABA response or biosynthesis and abiotic stress. Expression of genes were analyzed by qRT-PCR after treating with ABA in the WT, *bZIP4*-SRDX and *bZIP4*-Ox lines. Two-week-old plants were treated with 100 mM ABA and sampled at 0 h, 1 h, 3 h, and 6 h after treatment. The expression level of *eIF4A1* was used as an internal control. The expression level in 0 h was set to 1.0. Data represent the mean ± standard deviation of three individual experiments. Different letters indicate significant differences among transgenic lines at each time point ($p < 0.05$, one-way ANOVA followed by Tukey's HSD test)

interaction between additional CaM or CML proteins with bZIP4 and on the interaction with other bZIPs concerning the calcium signaling pathway via CaM could elucidate previously elusive or unknown associations.

In conclusion, as we propose in Fig. 7, in the normal condition of low level of Ca^{2+}, bZIP4 binds to its target promoters and enhances the expression of ABA-negative regulator genes. On the other hand, in stress conditions, cytosolic Ca^{2+}

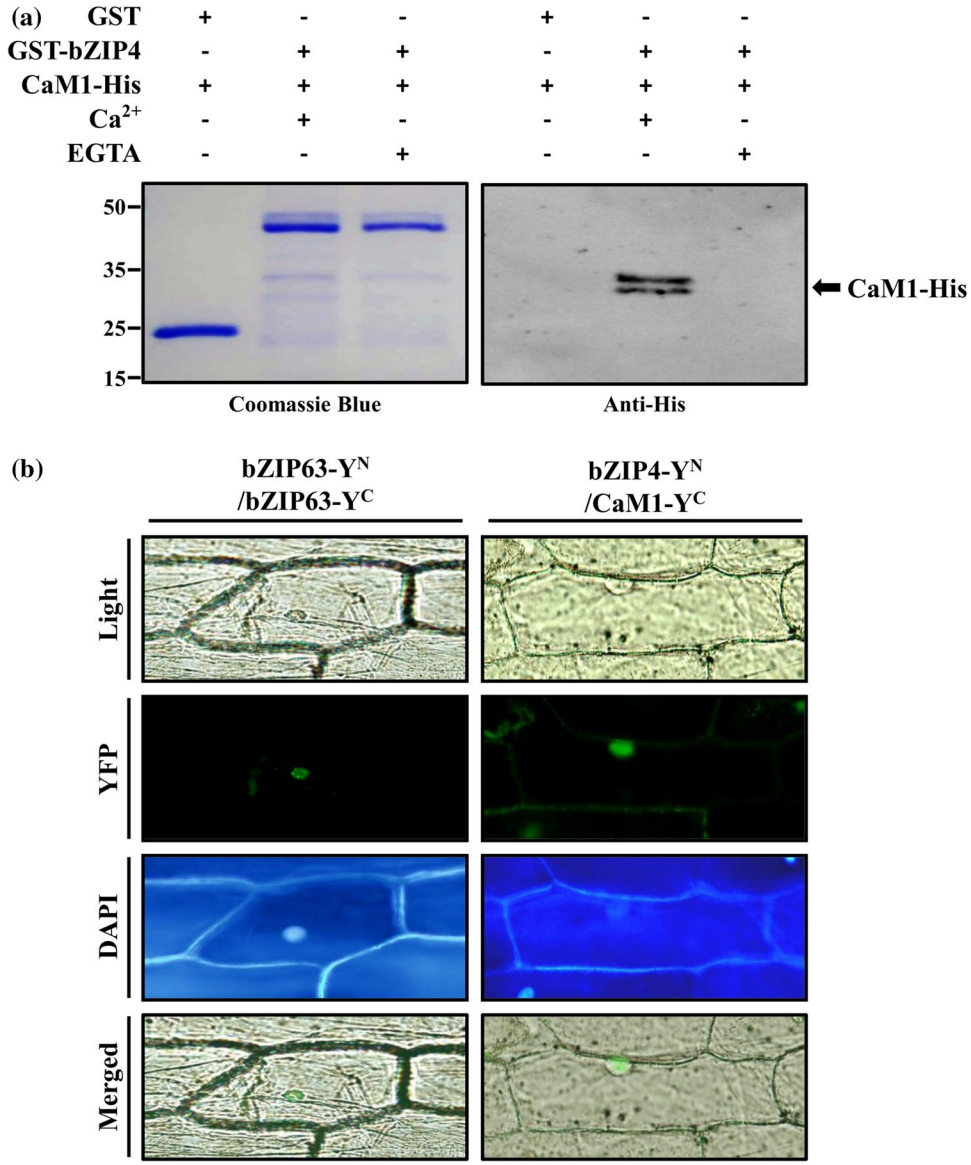

Fig. 5 Direct interaction of bZIP4 with CaM1 in vivo and in vitro. **a** Pull-down assay of bZIP4 with CaM1. GST-bZIP4 and CaM1-His were incubated in the presence (1 mM $CaCl_2$) or absence (5 mM EGTA) of Ca^{2+}. CaM1-His was pulled-down by GST (first lane, control), bZIP-GST with Ca^{2+} (second lane), and GST-bZIP4 without Ca^{2+} (third lane). The left panel is a polyacrylamide gel stained with Coomassie brilliant blue. The right panel is the result of western blot analysis for the pull-down assay. **b** BiFC of bZIP4 and CaM1 in vivo. bZIP63-YFP^C/bZIP63-YFP^N constructs were used as positive control. Light, light image; YFP, yellow fluorescent protein; DAPI, DAPI fluorescence image; Merged, merged image of YFP and DAPI

concentration is rapidly increased by its transport from the stored organelles as a response to the stress, activating CaM1 by binding to it. Activated CaM1 then binds to bZIP4, and this interaction could make bZIP4 to dissociate from promoters of genes encoding ABA-negative regulators, allowing the plants to operate on a typical ABA signaling pathway. However, further investigations are needed to elucidate the detailed mechanism(s) of bZIP4 in ABA signaling.

Materials and Methods

Plant Materials and Growth Conditions

Arabidopsis thaliana, ecotype Columbia-0, plants were used for our experiment. Plants were grown in soil (Sunshine Mix #5; Sun Gro Horticulture) at 22 ℃ and 60% humidity in a growth room under long-day conditions (16/8 h, light/dark). For in vitro experiments, plants were grown on half-strength MS medium (Duchefa) (Murashige and Skoog 1962) at 23 ℃ and 60% humidity in a growth chamber under long-day conditions.

Plant Abiotic Stress Treatment

Arabidopsis WT plants were grown for 2 weeks prior to being used for abiotic stress treatments. For ABA treatments, plants were sprayed with 100 mM ABA solutions. For drought treatment, leaves were detached from plants and were put on a filter paper under light at 25 ℃. For cold treatment, plants were transferred to a growth chamber at 4 ℃ for

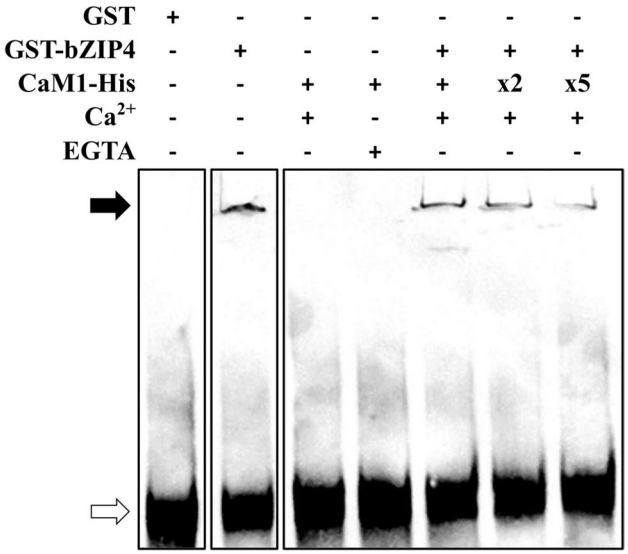

Fig. 6 Effect of CaM1 on DNA-binding affinity of bZIP4. EMSA of bZIP4 was performed with gradually increasing CaM1 concentrations. GST-bZIP4 was incubated with C-box element probes in the presence of CaM1 with Ca^{2+} (1 mM $CaCl_2$) or EGTA (5 mM EGTA). Black arrow indicates the shifted bands. White arrow indicates free probes

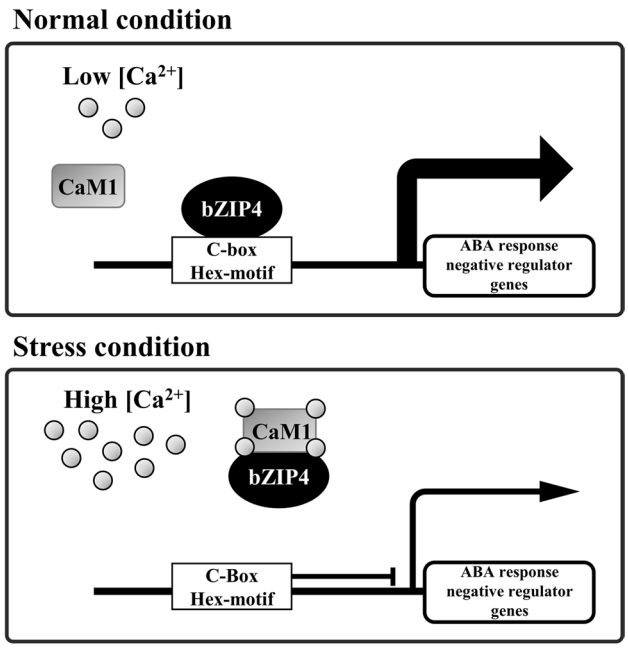

Fig. 7 A proposed model for bZIP4 in ABA regulatory pathway. In the normal condition of low calcium ion concentration, bZIP4 binds to its target promoters and enhances the expression of ABA-negative regulator genes. In stress conditions, however, cytosolic calcium ion concentration is rapidly increased by its transport from the stored organelles as a response to the stress, activating CaM1 by binding to it. Activated CaM1 then binds to bZIP4, and this interaction makes bZIP4

up to 12 h. Samples were taken at different time points after treatments, and then frozen by liquid nitrogen and stored at – 80 ℃ until being used for RNA extraction.

Root Length Measurement Under Stress Conditions

Seeds of the WT, *bZIP4*-Ox lines (9–4, 18–2), and *bZIP4*-SRDX Arabidopsis lines (14–1, 15–1) were surface-sterilized and then placed at 4 °C for 3 days in the dark prior to germination. Seeds were cultured on half-strength MS medium for 7 days, and then seedlings were transferred and cultured on half-strength MS medium supplemented with NaCl (50 and 100 mM) or glucose (2 and 4%) for 7 days. The length of roots was measured in three replicates.

Seed Germination Assay Under Stress Conditions

Seeds of the WT, *bZIP4*-Ox lines (9–4, 18–2), and *bZIP4*-SRDX lines (14–1, 15–1) were surface-sterilized and then placed at 4 °C for 3 days in the dark prior to germination. Seed germination was observed on half strength MS medium supplemented with NaCl (100, 150, and 200 mM) or glucose (2, 6, and 9%) at 23 °C and 60% humidity under long-day conditions. Seeds were considered to have germinated when the radicle protruded through the seed coat. The rate of seed germination was evaluated daily for 7 days. The germination rate was calculated as a percentage of the total number of seeds plated. Assays were carried out in three replicates of 30 seeds each.

Quantitative Reverse Transcription-PCR (qRT-PCR)

Total RNA was extracted using RNAiso plus (Takara Bio Inc.). A 1-μg sample of total RNA was reverse-transcribed with the oligo(dT) primer using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) with a 2-step RT-PCR protocol (60 min at 42 °C, 5 min at 70 °C). Quantitative real-time PCR was performed using a LightCycler® Real-Time PCR system (Roche Diagnostics). Each 20-ng cDNA sample was amplified with primers listed in Table S1 in triplicate using the KAPA SYBR® FAST qPCR kit (KAPA Biosystems). The *eIF4A1* gene was used as an internal control to normalize the differences in the amount of mRNA in each reaction. The PCR conditions consisted of 45 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 20 s. Ct (threshold cycle number) was determined by analysis with LightCycler® 480 II software. The relative gene expression was calculated using the WT or mock plants as the control, and using the equation $Y = 2^{-ddCt}$, where ddCt = test gene ($Ct_{sample} - Ct_{control}$) – normalization gene ($Ct_{sample} - Ct_{control}$).

Subcellular Localization Using Particle Bombardment

For the subcellular localization analysis, the *bZIP4* coding sequence except stop codon was amplified by PCR and digested with *Xba*I and *Bam*HI, then inserted into the corresponding site of pCAMBIA1300 binary vector that contains a green fluorescence protein (GFP)-coding sequence (Cui et al. 2013) with the CaMV 35S promoter and NOS terminator to yield the smGFP fusion protein (*Pro35S:bZIP4-smGFP*). GFP-coding sequence only with the CaMV 35S promoter and NOS terminator (*Pro35S:smGFP*) was used as a control. The construct was introduced into onion epidermal cells through particle bombardment using Biolistic® PDS-1000/Helium particle delivery system (Bio-Rad) with 1100 psi rupture disks and 1.0 µm gold microcarriers. Onion epidermis cells bombarded were incubated at 23 °C, under dark condition for 24 h and then GFP signals were observed with BX-51 fluorescence microscope (Olympus).

Construction of *ProbZIP4:GUS* Expression Plants and Histochemical Analysis

The promoter region of *bZIP4* (*ProbZIP4*) was amplified from Arabidopsis genomic DNA by PCR, digested with *Sal*I and *Bam*HI, and then inserted into the corresponding site of pBI101 vector (Clontech) containing beta-glucuronidase (GUS) gene. This construct was transformed into *Agrobacterium tumefaciens* GV3101 and then introduced into Arabidopsis (Koncz and Schell 1986) by the floral dipping method (Clough and Bent 1998). Transgenic lines were selected on half-strength MS medium containing 250 mg/L carbenicillin and 50 mg/L kanamycin, and survived plants were transferred to soil. Three independent T_3 homogeneous lines were selected and used for further study. For GUS histochemical staining, samples were incubated overnight with X-Gluc solution (1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronate, 10 mM Na_2EDTA, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.1% (v/v) Triton X-100, 50 mM $NaPO_4$, pH 7.0) at 37 °C. After staining, the chlorophyll-containing tissues were cleared in an ascending series of ethanol (50, 70 and 100%) and then observed under a microscope (BX-51 Fluorescence Microscope, Olympus).

Construction of *bZIP4* Overexpression and Repression Lines

To construct *Pro35S:bZIP4* overexpression vector, the coding region of *bZIP4* was amplified from Arabidopsis cDNA, digested with *BamH*I and *Sal*I, and then inserted into the corresponding site of a pCAMBIA1300 vector harboring CaMV 35S promoter and NOS terminator. To construct *Pro35S:bZIP4*-SRDX repression vector, a dominant repressor encoding the SRDX motif (LDLDLELRLGFA) was added to the 3′ end of the *bZIP4* coding region by replacing *bZIP4* stop codon (Mahfouz et al. 2012), and then cloned into pCAMBIA2300 having CaMV 35S promoter and NOS terminator as described by Cui et al. (2013). *Agrobacterium*-mediated transformation was performed as explained above. Transgenic lines were screened on half-strength MS medium containing 100 mg/L carbenicillin with 30 mg/L hygromycin for pCAMBIA1300 or 50 mg/L kanamycin for pCAMBIA2300, and then survived plants were transferred to soil. Two T_3 homogeneous lines were selected for further study.

Extraction and Purification of Recombinant Protein

The coding sequence of CaM1 was amplified, digested with *BamH*I and *Sal*I, and then cloned into pET-32b to yield a poly-histidine (His)-tagged protein (CaM1-His). The coding sequence of *bZIP4* was amplified, digested with *BamH*I and *Sal*I, and then cloned into pGEX-4T3 to yield a glutathione S-transferase (GST)-tagged protein (GST-bZIP4). These constructs were transformed into *Escherichia coli* BL21 CodonPlus® (DE3) RIL (Agilent Technologies). Expression of the recombinant proteins was induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 6 h at 30 °C. His-tagged and GST-tagged recombinant proteins were purified by gravity flow purification using Ni–NTA agarose (QIAGEN) and glutathione sepharose 4B (GE healthcare Life Sciences), respectively, as the affinity resin in Poly-Prep Chromatography columns (Bio-Rad).

Electrophoretic Mobility Shift Assay (EMSA)

To construct probes for the EMSA, C-box and Hex-motif elements were used (Table S1). The Biotin 3′ End DNA Labeling kit (Thermo Scientific) was used for labeling. EMSA of bZIP4 with or without CaM1 was performed as described by Nguyen et al. (2016).

Pull-Down Assay

For pull-down analysis of bZIP4 and CaM1, bZIP4-GST (3 mg) was conjugated to glutathione sepharose 4B bead (GE healthcare Life Sciences) and incubated with CaM1-His in a binding buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT, and 0.1% Triton X-100) with or without Ca^{2+} for 3 h at 4 °C. $CaCl_2$ was used to supply Ca^{2+}. Ethylene glycol tetraacetic acid (EGTA) was used to remove Ca^{2+} through chelation. Proteins were separated in an SDS–polyacrylamide gel and analyzed by immunoblotting, as described by Kim et al. (2017). The chemiluminescence signals were detected using the ImageQuant LAS 4000 mini (GE healthcare Life Sciences).

Bimolecular Fluorescence Complementation (BiFC) Assay

bZIP4 and CaM1 cDNAs were cloned in-frame into the *Bam*HI-*Sal*I sites of the pUC-SPYNE and pUC-SPYCE, named *Pro35S:bZIP4-YFP*^{N} and *Pro35S:CaM1-YFP*^{C}, respectively. The constructs were introduced into onion epidermal cells by particle bombardment using Biolistic® PDS-1000/Helium particle delivery system (Bio-Rad) with 1100 psi rupture disks and 1.0 μm gold microcarriers. Onion epidermis cells bombarded with *Pro35S:bZIP4-YFP*^{N} and *Pro35S:CaM1-YFP*^{C} were incubated in the dark for 24 h at 23 °C and then observed under BX-51 fluorescence microscope (Olympus).

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Declarations

Conflict of Interest The authors declare no conflicts of interest.

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