

# *GsERF6*, an ethylene-responsive factor from *Glycine soja*, mediates the regulation of plant bicarbonate tolerance in *Arabidopsis*

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## Abstract

**Main conclusion** This is an original study focus on ERF gene response to alkaline stress. *GsERF6* functions as transcription factor and significantly enhanced plant tolerance to bicarbonate ( $\text{HCO}_3^-$ ) in transgenic *Arabidopsis*.

Alkaline stress is one of the most harmful, but little studied environmental factors, which negatively affects plant growth, development and yield. The cause of alkaline stress is mainly due to the damaging consequence of high concentration of the bicarbonate ion, high-pH, and osmotic shock to plants. The AP2/ERF family genes encode plant-specific transcription factors involved in diverse environmental stresses. However, little is known about their physiological functions, especially in alkaline stress responses. In this study, we functionally characterized a novel ERF subfamily gene, *GsERF6* from alkaline-tolerant wild soybean (*Glycine soja*). In wild soybean, *GsERF6* was rapidly induced by  $\text{NaHCO}_3$  treatment, and its overexpression in *Arabidopsis* enhanced transgenic plant

tolerance to  $\text{NaHCO}_3$  challenge. Interestingly, *GsERF6* transgenic lines also displayed increased tolerance to  $\text{KHCO}_3$  treatment, but not to high pH stress, implicating that *GsERF6* may participate specifically in bicarbonate stress responses. We also found that *GsERF6* overexpression up-regulated the transcription levels of bicarbonate-stress-inducible genes such as *NADP-ME*, *H<sup>+</sup>-Ppase* and *H<sup>+</sup>-ATPase*, as well as downstream stress-tolerant genes such as *RD29A*, *COR47* and *KINI*. *GsERF6* overexpression and  $\text{NaHCO}_3$  stress also altered the expression patterns of plant hormone synthesis and hormone-responsive genes. Conjointly, our results suggested that *GsERF6* is a positive regulator of plant alkaline stress by increasing bicarbonate ionic resistance specifically, providing a new insight into the regulation of gene expression under alkaline conditions.

**Keywords** Abscisic acid · Alkali stress · ERF transcription factor · Ethylene · Functional analysis · Wild soybean

## Abbreviations

ABA	Abcisic acid
ET	Ethylene
JA	Jasmonate
OX	Overexpression
TF	Transcription factor
WT	Wild type

## Introduction

Adverse environmental conditions, such as soil saline-alkalinity, drought, low temperature, and mineral deficiency severely affect crop yield and quality worldwide, resulting in great economic losses. Plants have developed complex

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mechanisms to deal with salt stress, such as the well-known SOS signaling pathway (Mahajan et al. 2008) and MAP kinase signal transduction (Nakagami et al. 2005). Comparatively little attention was paid to alkaline stress, and not much information was reported about how plants respond to alkaline stress, although it imposes much more severe damage than other stresses on plant growth and development (Yang et al. 2008).

Alkaline soils are usually classified by low availability of nutrients, high concentration of  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$ , and high pH (Misra and Tyler 1999). Bicarbonate ( $\text{HCO}_3^-$ ) and carbonate ( $\text{CO}_3^{2-}$ ) are the principal contributors to alkalinity, whereas hydroxide, borate, ammonia, organic bases, phosphates, and silicates are considered as minor contributors (Petersen 1996). Many common commercial crops are reported to be severely affected by  $\text{HCO}_3^-$  (Hajiboland et al. 2005; Valdez-Aguilar and Reed 2010; Rouphael et al. 2010). The bicarbonate also reduces the uptake and utilization of iron ( $\text{Fe}^{2+}$ ), leading to Fe deficiency and lime-induced leaf chlorosis (Chaney et al. 1992). Some early studies compared the effects of neutral salts ( $\text{NaCl}$  or  $\text{Na}_2\text{SO}_4$ ) and alkaline salts ( $\text{NaHCO}_3$  or  $\text{Na}_2\text{CO}_3$ ) on growth, photosynthesis, ion balance and osmotic adjustment in different species of plants such as barley (Yang et al. 2009), cotton (Chen et al. 2011), *Chenopodium glaucum* (Shasha 2012) and wheat (Guo et al. 2015), aiming to elucidate the mechanisms of alkali stress damage and the physiological adaptive mechanisms of plants to alkali stress. High concentration of  $\text{NaCl}$  (>300 mM) seriously delayed seed germination of *C. glaucum*, however, the inhibitory effect of  $\text{NaHCO}_3$  stress on radicle and hypocotyl elongation was greater than  $\text{NaCl}$  stress even at lower concentration (100 mM) (Shasha 2012).  $\text{NaHCO}_3$  stress also inhibits the absorption of some inorganic anions such as  $\text{H}_2\text{PO}_4^-$ ,  $\text{Cl}^-$  and  $\text{NO}_3^-$ , which greatly decreases the selective absorption ratio of  $\text{K}^+/\text{Na}^+$ , and breaks the ionic balance (Yang et al. 2007). Organic acids and  $\text{SO}_4^{2-}$  might play important roles to maintain intracellular ion balance and pH stabilization under alkali stress, while, inorganic ions are major contributors to ion balance under salt stress (Yang et al. 2009). Moreover, the seedlings exposed to  $\text{NaHCO}_3$  generated higher levels of reactive oxygen species (ROS,  $\text{O}_2^-$ ,  $\text{H}_2\text{O}_2$ ), and showed increased activities of antioxidant enzymes (SOD, POX) even at lower concentration of  $\text{NaHCO}_3$  (Shasha 2012). All of these studies suggest the alkaline salts could generate more severe effects on plants than the neutral salts, and the researchers speculate it might be due to the additive influence of high pH. Until now, most studies about alkaline stress have focused on the physiological impact on plants, while its molecular mechanism is rarely reported.

Thus, understanding the molecular and physiological mechanism of plant responses to alkaline stress and mining key regulatory genes in the stress responses are very

important for crop improvement. As a typical salt and alkaline resistant species, wild soybean *Glycine soja* G07256 was used as a model plant in this study. We have reported a comprehensive and pioneer transcriptome analysis of wild soybean root given by salt and alkaline challenges using Affymetrix<sup>®</sup> Soybean GeneChip<sup>®</sup> (Ge et al. 2010), and functionally characterized some key regulatory genes in alkaline stress responses, such as *GsCBRLK* and *GsTIFY10* (Yang et al. 2010; Zhu et al. 2011). Using high throughput RNA sequencing technique, we sequenced the transcriptome of *G. soja* roots under  $\text{NaHCO}_3$  stress on the Illumina Genome Analyzer IIx (GAIIx) platform (DuanMu et al. 2015), providing a broad description of gene expression pattern and gene transcriptional dynamics under alkaline stress in *G. soja*. One of the intriguing candidates is an AP2/ERF transcription factor (TF) which was highly induced by  $\text{NaHCO}_3$  and may be a putative alkali-responsive gene.

The AP2/ERF family is a large and plant-specific transcription factor family. It can be further classified into four major subfamilies (AP2, DREB, ERF and RAV) based on the similarity of their conserved domains. AP2 subfamily members contain double AP2/ERF domains, and RAV subfamily members contain one AP2/ERF domain and an additional B3 DNA-binding domain, while DREB and ERF subfamily members contain only one single AP2/ERF domain (Sakuma et al. 2002). The DREB and ERF subfamilies can be divided into six subgroups, A-1 to A-6 and B-1 to B-6 (Nakano et al. 2006), respectively. In *Arabidopsis*, an ERF subfamily gene *RAP2.2* can be induced by hypoxia in an ethylene dependent manner, and overexpression or knockout of *RAP2.2* resulted in improving or impairing the survival of plants under hypoxic stress, respectively, and *RAP2.2* and its downstream genes have a transcriptional cascade and play an important role under hypoxic stress or submergence conditions (Hinz et al. 2010; Bui et al. 2015). A recent study about ERF6 protein in *Arabidopsis* has shown that ERF6 can modulate oxidative gene expression by interacting with MPK6 (Wang et al. 2013). The expression of *ERF6* can be rapidly induced by pathogen, SA, ROS and cold stress but suppressed by water deficit, heat and ABA (Wang et al. 2013). These data indicate that ERF6 has important roles and is required in biotic and abiotic stress signaling during plant growth and development.

In recent years, the identification of some soybean ERF genes and their roles in abiotic stress tolerance has been attempted. A member of ERF family from *Glycine max* has been reported to function as a transcriptional activator of downstream defense genes in both soybean and *Arabidopsis*, and mediates the expression of defense-related genes regulated by ethylene (ET), jasmonate (JA) and salicylic acid (SA) (Mazarei et al. 2007). Moreover, *GmERF3* also functions as a transcriptional activator

activating the expression of reporter genes and is able to bind with both the GCC box and DRE/CRT motifs in yeast cells. Overexpression of *GmERF3* can influence plant resistance to both biotic and abiotic stresses such as bacterial, fungal, viral pathogens, salt and drought stresses (Zhang et al. 2009). Similarly, expression of *GmERF4* could be induced by both biotic stimuli such as ethylene, JA, SA, soybean mosaic virus and abiotic stresses such as cold, salt and drought, but repressed by ABA. Constitutive expression of *GmERF4* in tobacco increased tolerance to salt and drought stresses but did not exhibit detectable tolerance to bacterial infection (Zhang et al. 2010).

However, little is known about responses of ERF genes to alkaline stress. In the present study, we isolated a putative AP2/ERF gene, *GsERF6* from wild soybean. Overexpression of *GsERF6* in *Arabidopsis* conferred deliberately enhanced tolerance to alkali stress, and up-regulated the expression levels of stress-responsive marker genes, and altered the expression levels of biosyntheses and responsive genes of endogenous hormones like ABA, ET and JA. Further analysis showed that *GsERF6* overexpression in *Arabidopsis* displayed particular response to  $\text{HCO}_3^-$  but not high pH stress, suggesting that *GsERF6* may play special roles in response to  $\text{HCO}_3^-$  stress. Subcellular localization and transcriptional activation studies showed that GsERF6 is a nuclear protein with transcriptional activation. All the data presented here illustrate the important role of GsERF6 as a regulator of alkali stress tolerance in plants.

## Materials and methods

### Plant material and stress treatment

The wild soybean line (G07256) was collected from Baicheng in Jilin Province, China as described (Ge et al. 2010). To analyze the expression pattern of *GsERF6* under alkaline stress, *G. soja* G07256 seedlings were grown in 1/4 strength Hoagland's solution in a culture room. The environmental conditions are as following: 24 °C, 60 % relative humidity and a light regime of 16 h light/8 h dark. The seeds were first shaken in 98 % sulfuric acid for 10–15 min to break dormancy, and then washed with sterile water for at least five times before being placed on wet filter papers in petri dishes in the dark for germination. After 2 days, germinating seeds were then transferred into the growth boxes containing 1/4 strength Hoagland's solution. The nutrient solution was changed every 3 days. The 21-day seedlings were moved into 1/4 strength Hoagland's solution with 50 mM  $\text{NaHCO}_3$  (pH 8.5). Equal amounts of roots were sampled at seven different time points: 0, 1, 3, 6, 9, 12 and 24 h for RNA extraction and qRT-PCR analysis. The root samples were snap frozen in liquid  $\text{N}_2$  and stored at  $-80$  °C.

*Arabidopsis thaliana* ecotype Columbia (Col-0) seeds obtained from Nottingham Arabidopsis Stock Centre (NASC) were grown in the chamber at the following conditions: 21–23 °C, 60 % relative humidity, 100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , 16 h light/8 h dark cycles. To analyze the expression of alkali stress-related genes in wild type (WT) and *GsERF6* overexpression (OX) lines, the seeds were germinated and grown on 1/2 MS agar plates for 12 days, and then the *Arabidopsis* plants were moved to the filter paper saturated with normal 1/2 MS solution (control) or 1/2 MS solution with 50 mM  $\text{NaHCO}_3$ . Samples were collected at 0, 6 and 12 h, respectively, after treatment.

### RNA isolation, cDNA synthesis and quantitative real-time PCR

Total RNA extractions were done using RNeasy Plant Kit (Qiagen Biotech, Beijing, China) and cDNA syntheses were performed by SuperScript<sup>TM</sup> III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) with Oligo(dT)18 reverse primer. qRT-PCR analyses were performed using SYBR Premix ExTaq<sup>TM</sup> II Mix (TaKaRa, Shiga, Japan). *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase, accession no. DQ355800) was used as reference in *G. soja*. *Actin2* (ACTIN2) was used as reference in *Arabidopsis*. The expression levels of all candidate genes were analyzed by the  $2^{-\Delta\Delta\text{CT}}$  method. The primers used for qRT PCR were listed in Supplementary Table S1.

### *GsERF6* gene isolation and sequence analysis

*GsERF6* was cloned by homology-based cloning method. *GsERF6* was amplified by PCR primer pairs 5'-GATGGC TAACGCTGCTGAAGT-3' and 5'-TTCGTCAAATGTAC AATGTACTCATC-3'. The PCR products were inserted into pEASY-Blunt Simple Cloning vector (Transgen, Beijing, China) and sent for sequencing. Phylogenetic analysis was performed using MEGA 5.0. The NJ tree was constructed by AP2/ERF homologs of *G. soja*, *Selaginella moellendorffii*, *Physcomitrella patens* and *Arabidopsis* based on the JTT model. Peptide sequence information was obtained from Phytozome (<http://phytozome.jgi.doe.gov/pz/portal.html>). Sequence alignments were performed with ClustalX.

### Protein subcellular localization analysis

To analyze subcellular localization of GsERF6, we inserted *GsERF6* gene in the 5' of *eGFP* gene in pBSKII-eGFP vector to express GsERF6-eGFP fusion protein in plant cells. The resultant pBSKII-GsERF6-eGFP or the empty plasmid were transformed into onion epidermis cells separately by the particle bombardment method as described

(Luo et al. 2013). The expression and localization of fluorescent proteins in the epidermis cells were imaged using a confocal laser-scanning microscope (SP5, Leica, Wetzlar, Germany).

### In vitro transcriptional activation assay

*GsERF6* and *AtDREB1A* were inserted into pGBKT7-BD vector. The resulted pGBKT7-*GsERF6*, pGBKT7-*AtDREB1A* or the empty plasmid was transformed into yeast strain AH109, respectively. The transformants were selected on solid SD/-Trp medium at 30 °C for 3 days. The grown cells from independent colonies were re-streaked on SD/-Trp medium for another 2 days, and then were collected for measuring  $\beta$ -galactosidase activity by colony-lift filter assay. At the same time, the independent colonies were picked and grown on SD/-Trp-His medium for another 3 days to evaluate the expression of HIS3 reporter gene. Transcriptional activation was analyzed according to the methods described on Yeast Protocols Handbook (Clontech).

### Histochemical GUS assay

To investigate *GsERF6* gene expression pattern in planta, approximately 2.5 kb of its promoter (−2572 to −14 upstream of the translation initiation codon) was amplified by PCR from wild soybean genomic DNA. The PCR product was inserted into the pCAMBIA 3301 vector between *EcoRI* and *BglII* sites in the upstream of GUS gene. The resulted construct or the empty vector was transformed into *Agrobacterium tumefaciens* strain LBA4404 and then into *Arabidopsis thaliana* by floral dip method as previously described (Clough and Bent 1998). Fifteen glyphosate-resistant transgenic ( $T_1$ ) plants were obtained and four independent  $T_3$  lines were analyzed.

For histologic analysis, the *Arabidopsis* seedlings were grown on 1/2 MS plates and soil separately. On the 2nd and 8th day after germination, three to four seedlings from each line on 1/2 MS plates were sampled and applied to GUS staining. On the other hand, the flowers and seed pods from 45-day-old plants grown on soil were sampled, and then subjected to GUS staining. GUS histochemical staining was performed using 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide (X-Gluc) as substrate (Thomine et al. 2003).

To determine the GUS expression pattern in the transgenic *Arabidopsis* plants in response to alkali stress, the seeds from four independent transgenic lines were germinated on 1/2 MS medium for 7 days, and then transferred to filter papers soaked with 1/2 MS liquid medium containing 50 mM NaHCO<sub>3</sub> for 6 or 12 h before GUS staining. Plants or tissues were then visualized using a stereo microscope (Olympus, Tokyo, Japan).

### Vector construction and generation of transgenic *Arabidopsis thaliana*

The full coding region of *GsERF6* was amplified from pEASY-*GsERF6* with *SmaI* and *XbaI* linker primers 5'-TG ACCCGGGATGGCTAACGCTGCTG-3' and 5'-TGCTCT AGATCACACAGCCACGAGCGGT-3', and then was cloned into the plant expression binary vector pCAMBIA2300 to generate pCAMBIA2300-*GsERF6*. The recombinant vector was transformed to *Agrobacterium tumefaciens* strain LBA4404 and then the targeted gene was transferred into *Arabidopsis* plants by floral dip method. The transgenic seedlings were selected on 1/2 MS medium plate containing 50 mg L<sup>-1</sup> kanamycin. The positive plants were transferred into soil and the mature seeds were individually harvested. The  $T_2$  seeds were selected again on kanamycin-containing 1/2 MS medium and the homozygous plants were determined and identified by genotyping the  $T_2$  seedlings on selective medium. The gene expression levels were confirmed by RT-PCR analysis. The primers used for RT-PCR were listed in Supplementary Table S2.

### Phenotypic analysis of transgenic *Arabidopsis* plants

The *Arabidopsis* seeds were firstly sterilized in 5 % NaClO solution for 5 min and washed with sterilized water for 5 times, and then were transferred onto wet filter paper and stored at 4 °C in the dark for 3–7 days. The plants were grown in a tissue culture room or a chamber under controlled environmental conditions of 21–23 °C, 100  $\mu$ mol photons m<sup>-2</sup> s<sup>-1</sup>, 60 % relative humidity and 16/8 h day-night cycles.

To analyze germination rates, the seeds of WT and *GsERF6* OX lines were sown on 1/2 MS agar plates supplemented with 0, 6 or 8 mM NaHCO<sub>3</sub>, or 6 or 7 mM KHCO<sub>3</sub>, or 1 M KOH to adjust the medium pH at 7.5–8.2. The germination rates were recorded for 6 consecutive days after sowing. Pictures were taken to show the growth performance of each line, followed by measurement of the opening/greening of the leaves. For each experiment, ninety seeds were used and all experiments were generated at least three replications.

To characterize stress tolerance at the early seedling stage, WT and OX *Arabidopsis* seeds were germinated and grown on 1/2 MS medium for 7 days, and then were transferred to plates with vertical position containing 1/2 MS agar in the absence or presence of 6 mM NaHCO<sub>3</sub>, or 5–7 mM KHCO<sub>3</sub>, or at pH 7.5–8.2 (pH was adjusted with 1 M KOH) for 7–10 days before photographed. To analyze stress tolerance at adult stage, the seedlings were grown in pots filled with the mixture of vermiculite:peat moss:garden soil (volume ratio, 1:1:1). The plants were irrigated with 100 mM NaHCO<sub>3</sub> solution every 4 days for a total of



16 days. The photos were taken at different stages of growth and development.

**Statistical analysis**

All experiments with each group were performed at least in triplicate. Data were reported as mean ± SD. Data were analyzed statistically by Duncan’s multiple range tests or Student’s *t* test. Results were considered statistically significant when *P* < 0.05.

**Results**

**Isolation and sequence analysis of *GsERF6***

AP2/ERF TFs demonstrate to be related to many regulation processes when plants respond to various environmental stresses. In this study, *GsERF6* was isolated from *G. soja* by homology-based cloning method according to the cDNA sequence of Glyma05g05130 from *G. max*. *GsERF6* contains a complete open reading frame (ORF) of 837 bp which is 99 % identical to Glyma05g05130 based on the genome database from Phytozome.

AP2/ERF family TFs are specifically evolved in plants and the evolutionary process of AP2/ERF proteins can be traced back to the lowest plant green alga *Chlamydomonas reinhardtii* (Shigyo et al. 2006). To know the phylogenetic relationship and classification of *GsERF6*, phylogenetic analysis was performed using MEGA 5.0 software based

on *GsERF6* sequence and other AP2/ERF proteins from *Physcomitrella patens*, *Selaginella moellendorffii* and *Arabidopsis* which represent mosses, lycophytes and angiosperms, respectively. *GsERF6* is clustered to the B-3 subgroup of ERF subfamily and is closely related to AT5G47230 (*AtERF5*) in the phylogenetic tree as shown in Supplementary Fig. S1.

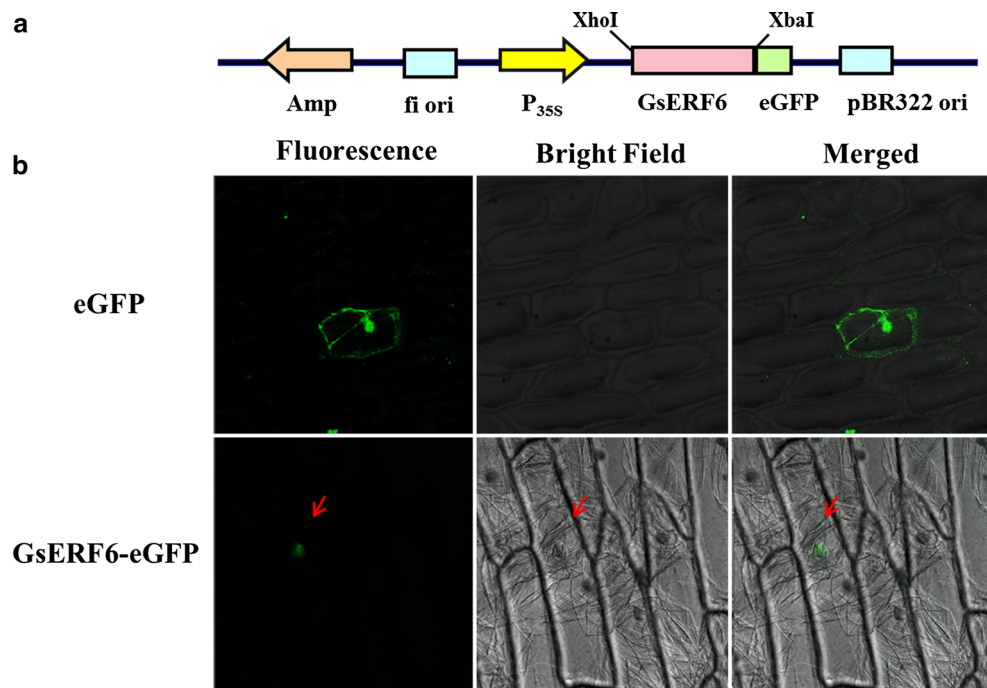
*GsERF6* contains one AP2 domain which is approximately 60 amino acids from 129 to 187 aa. Its AP2 domain is highly conserved compared with the other plant ERF proteins (Supplementary Fig. S2). Further structural analysis of *GsERF6* protein showed that the conserved AP2 domain contains a three-strand β-sheet which is very important to recognize the target sequence and a C-terminal α-helix, which is similar with the structure of *Arabidopsis* ERF1 protein (Allen et al. 1998). This suggests that *GsERF6* has ability to bind with ERE-like (ethylene response element-like) DNA sequences.

***GsERF6* is a nuclear protein with transactivation activity**

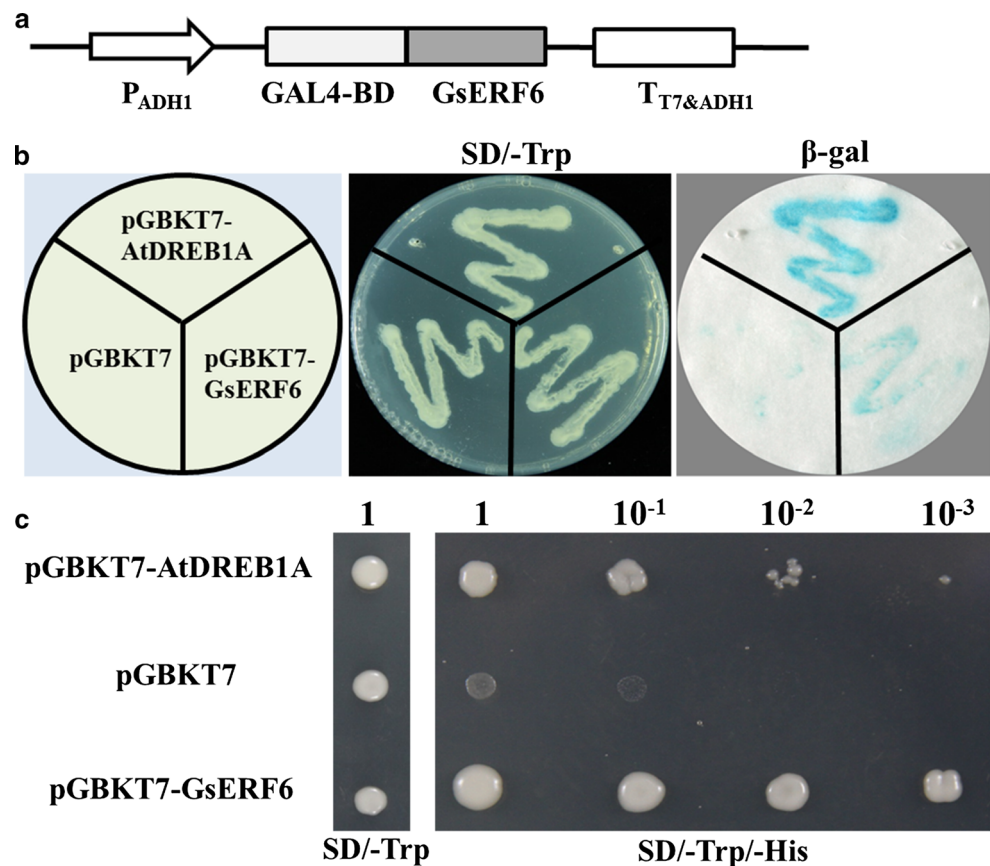
Transcription factors are a kind of proteins functioning as activators or repressors to regulate gene expression or transcription. To investigate the transcription activity of *GsERF6* protein, we need firstly determine its subcellular localization. *GsERF6* coding sequence without stop codon was fused at the 5' end of *eGFP* (Fig. 1a) and the resultant construct was transformed into plant cells. The results of confocal imaging showed that *GsERF6*-*eGFP* fusion

**Fig. 1** Subcellular localization of the *GsERF6* protein.

**a** Schematic representation of constructs used for subcellular localization analysis of *GsERF6*. **b** Images of onion epidermis cells expressing *eGFP* only (*upper lane*) and *GsERF6*-*eGFP* fusion protein (*bottom lane*). The subcellular localization was examined by confocal microscopy under fluorescent-field illumination, bright-field illumination and an overlay of bright and fluorescent illumination, respectively



**Fig. 2** Transcription activation activity of GsERF6 in yeast. **a** Schematic representation of constructs used for transactivation activity analysis of GsERF6. **b, c** Transcriptional activation analysis of GsERF6 in yeast cells. pGBKT7-*AtDREB1A* and pGBKT7 were used as positive and negative controls, respectively



protein was localized in the nuclei of onion epidermal cells by a transient expression assay (Fig. 1b). As a control, eGFP protein alone was found in both the nucleus and cytoplasm. The nuclear localization of GsERF6 suggested that this protein may play a role as a transcription factor.

The transcriptional activation activity of GsERF6 was investigated in yeast cells. First *GsERF6* was inserted into pGBKT7-BD vector to fuse with GAL4 DNA-binding domain coding sequence (Fig. 2a) and then transformed to yeast AH109 strain with positive and negative controls. The colonies carrying the relevant plasmids were selected on SD/-Trp plates. The randomly selected colonies were used to measure the transcription of LacZ reporter gene using the colony-lift filter assay to test activation ability of GsERF6. As shown in Fig. 2b, the positive control exhibited strong  $\beta$ -galactosidase activity but the negative control did not. The transformants carrying pGBKT7-GsERF6 showed a little lower  $\beta$ -galactosidase activity than the positive but much stronger than the negative (Fig. 2b), indicating that GsERF6 may have transcriptional activation ability in yeast cells. Furthermore, we dropped a serial of diluted yeast cell suspensions of representative colonies from SD/-Trp medium onto SD/-Trp-His medium to see if GsERF6 can activate *HIS3* reporter gene. As shown in Fig. 2c, all yeast cell lines grew similarly on SD/-Trp

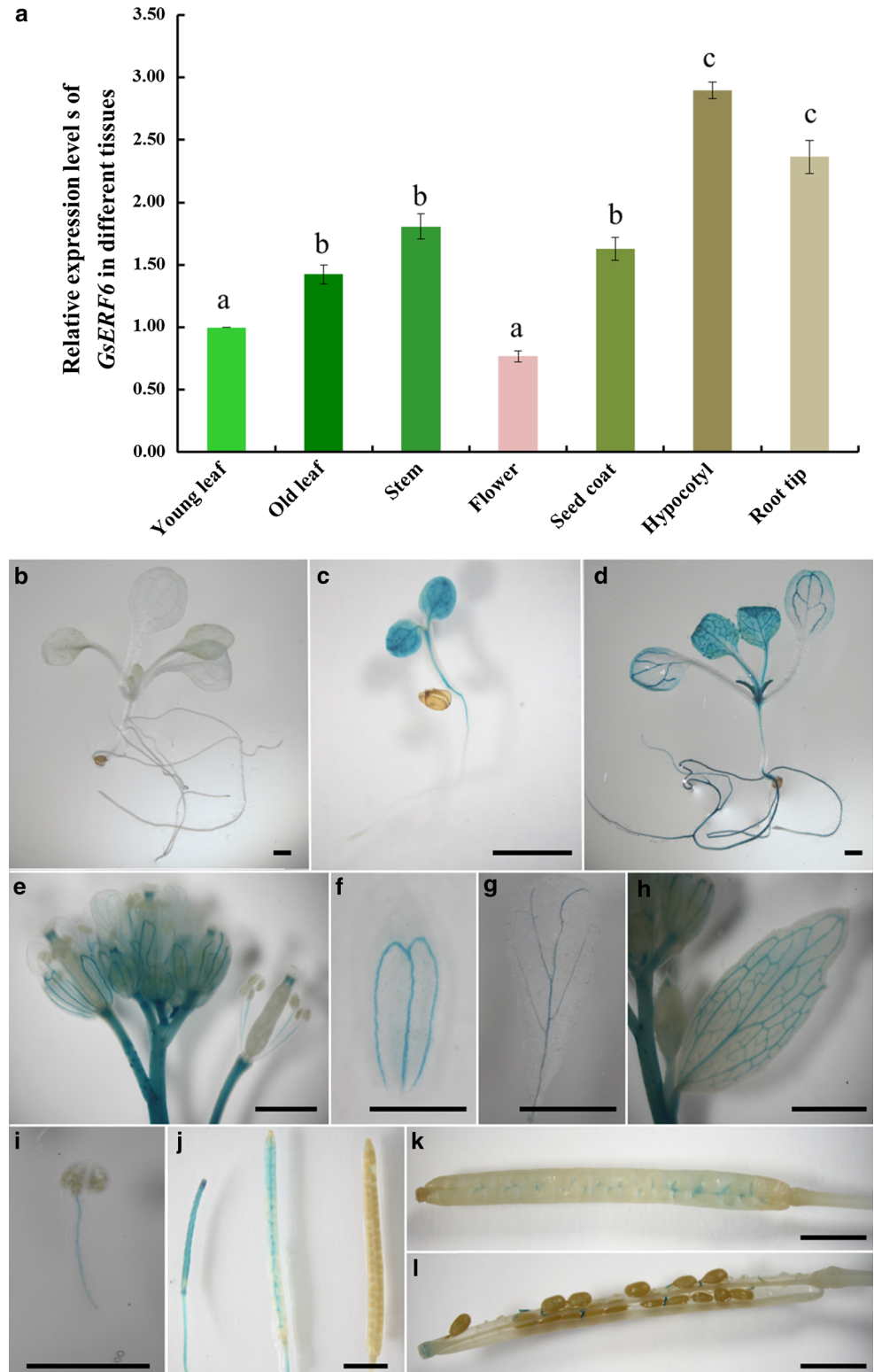
medium, but differently on SD/-Trp-His medium. The cells carrying GsERF6 grew almost as well as the positive control cells but the negative cells had no growth (Fig. 2c), suggesting GsERF6 can also activate *HIS3* reporter gene as well. All of these data indicated that GsERF6 may be a putative transcription factor possessing transcriptional activation activity.

### Spatial expression pattern of *GsERF6*

To investigate the spatial expression of endogenous *GsERF6* in *G. soja* 07256, qRT-PCR was performed to analyze *GsERF6* expression in different tissues including young leaves, old leaves, stems, root tips, hypocotyl and seed coats from mature *G. soja* 07256 seedlings. The qRT-PCR data showed *GsERF6* was widely expressed in all tissues, but highly expressed in hypocotyls and roots with more than twofold in leaves (Fig. 3a).

To further determine *GsERF6* promoter activity, we imaged representative GsERF6pro-driven GUS spatial expression patterns in transgenic *Arabidopsis* plants. The GUS activity was observed in all tissues, including root, leaf, stem, flowers and immature siliques (Fig. 3c–l). In most tissues, strong GUS expression was monitored in vasculatures, especially in young tissues. While, in

**Fig. 3** Spatial expression pattern analysis of *GsERF6*. **a** Tissue-specific expression patterns of *GsERF6* were examined in young leaf, old leaf, stem, flower, seed coat, hypocotyl and root from *G. soja*. Data were analyzed by Duncan’s multiple range tests. The mean value from three fully independent biological repeats and three technical repeats is shown. **b–l** Histochemical analyses for GUS expression in *Arabidopsis thaliana* transgenic plants under the control of *GsERF6* promoter. **b** Control *Arabidopsis* with six leaves. **c**, **d** Young plants (2 and 10 days after germination). **e** Inflorescence. **f** Sepal. **g** Petal. **h** Cauline leaf below flowers. **i** Stamen. **j** Immature siliques. **k–l** Funiculus and micropilar endosperm. Scale bars 2 mm



young seedlings, the expression was mainly in the vascular bundles of leaves, cotyledons, hypocotyls and in the central cylinder of primary and secondary roots, but not found in root tips (Fig. 3c, d). In flowers, the

staining was mainly observed in vascular bundles of sepal, petal, stigma and filament (Fig. 3e–i). Young siliques had more GUS expression than mature ones. In mature siliques (Fig. 3k, l), GUS activity decreased



significantly but majorly expressed at hilum, where the only nutrition transport corridor from vegetative organs to seeds is. The tissue localization results indicated that *GsERF6* may play important roles during the process of nutrient and mineral acquisition, absorption and transportation in the plant.

### Expression of *GsERF6* under $\text{NaHCO}_3$ treatment

Our high throughput RNA seq data indicated that *GsERF6* was highly induced in the roots of alkali-stressed *G. soja* (DuanMu et al. 2015). To determine the expression pattern of *GsERF6* in response to alkali stimuli, we extracted total RNA from *G. soja* 07256 roots and used RT-PCR to measure *GsERF6* expression levels from different treatments. Under alkali stress, *GsERF6* transcription level was first reduced slightly at the 1 h point after treatment, and then increased rapidly and peaked at 6 h with a fivefold increase, and finally, the expression of *GsERF6* came down to the normal level at 24 h (Fig. 4a). Nevertheless, the temporal *GsERF6* promoter-driven GUS activity in *Arabidopsis* also showed dynamic change within the period of the treatment (Fig. 4b). Collectively, *GsERF6* might be an important regulatory gene in alkaline-response pathway.

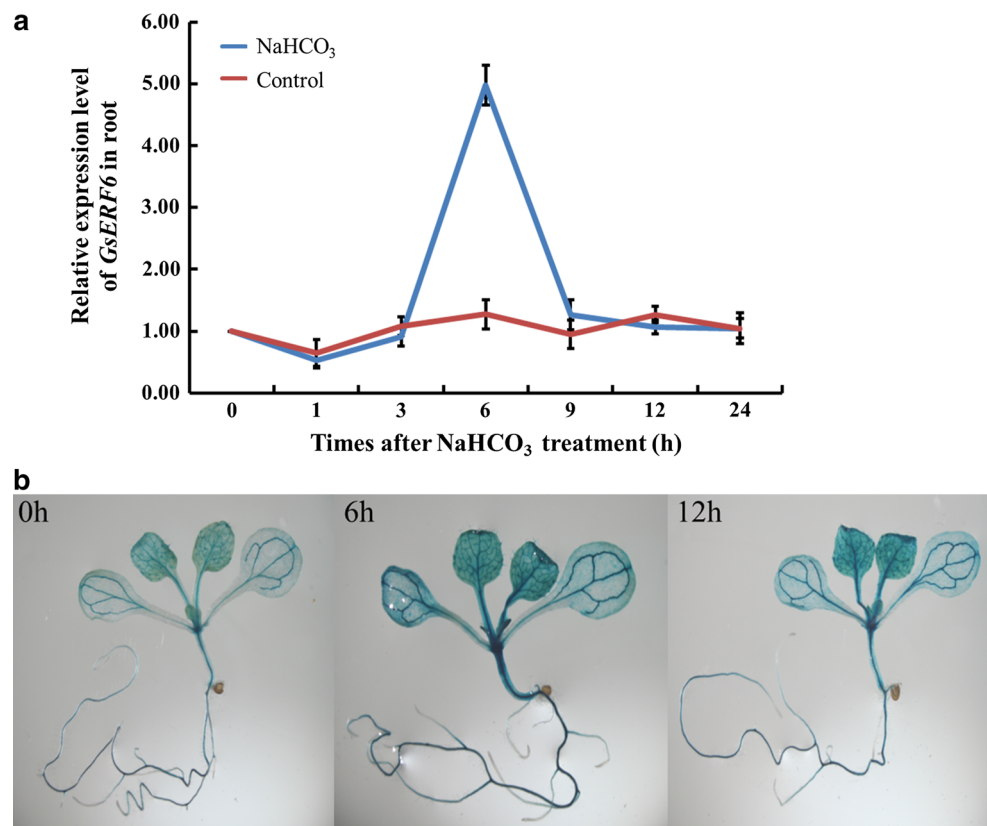
We then analyzed the upstream sequence of *GsERF6* by a MEME search. The result revealed that some stress- and

defense-related elements, such as HSEs (position  $-258$  to  $-267$  and  $-1592$  to  $-1601$ ), LTR (positions  $-1281$  to  $-1286$ ), MBSs (position  $-386$  to  $-391$ ), TC-rich repeats (position  $-412$  to  $-421$ ,  $-673$  to  $-682$  and  $-952$  to  $961$ ) and WUN-motif (positions  $-600$  to  $-609$ ) were enriched, implicating that *GsERF6* is a stress-related gene. However, no other well-known motif was found, suggesting that there may be unknown *cis*-element(s) in the promoter leading to alkali-inducible transcription of *GsERF6*.

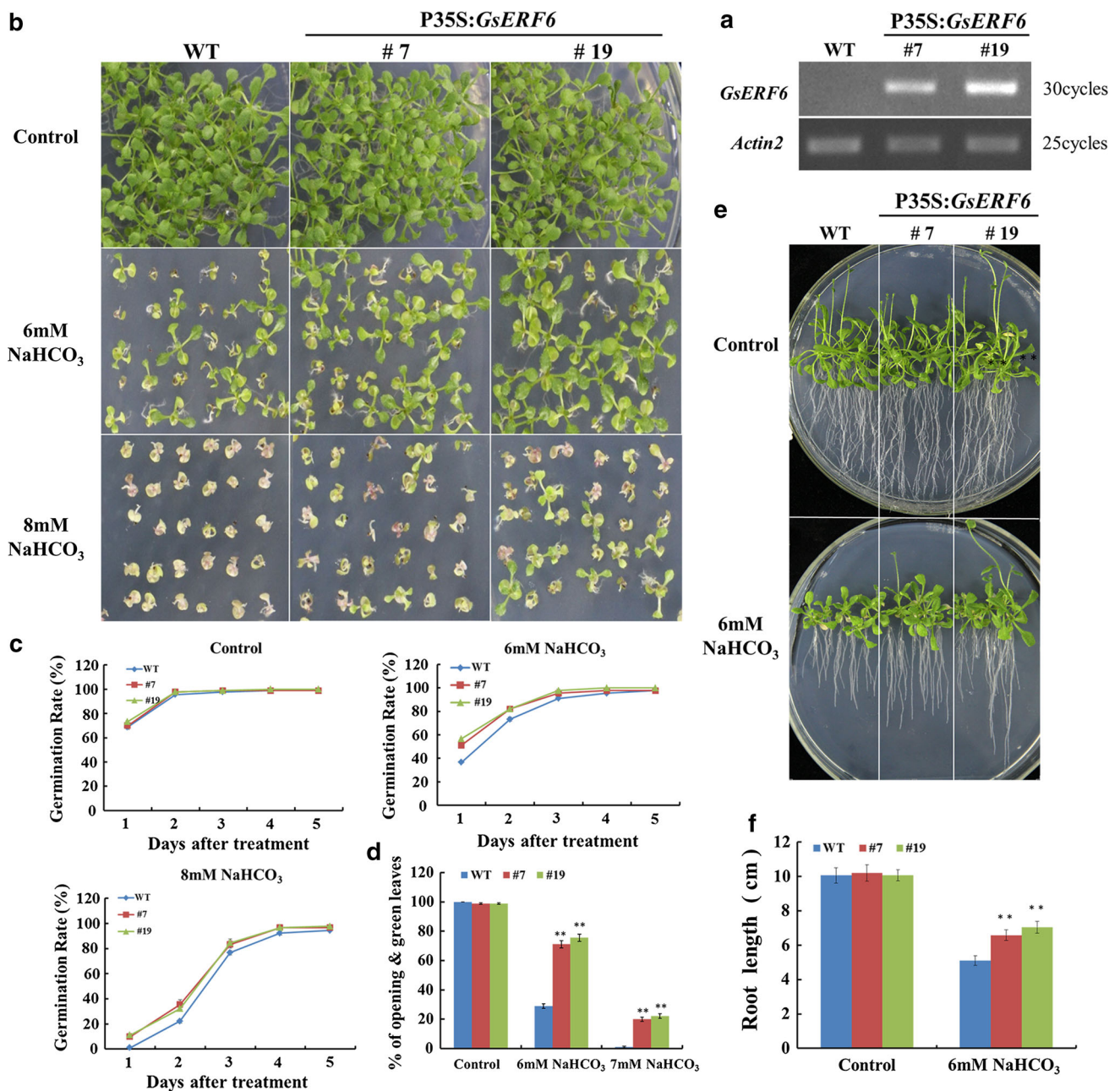
### Overexpression of *GsERF6* enhanced plant $\text{NaHCO}_3$ tolerance

To determine the regulatory role of *GsERF6* under  $\text{NaHCO}_3$  treatment *in planta*, we generated transgenic *Arabidopsis* lines carrying CaMV35S-driven *GsERF6* constitutive overexpression (OX) cassette. Two homozygous T<sub>3</sub> OX *Arabidopsis* lines (#7, #19) with relatively high *GsERF6* expression level were selected for further analysis. Compared with the untransformed wild type (WT) plants, the increased transcription levels of *GsERF6* in the OX lines were confirmed by semi-quantitative RT-PCR analysis (Fig. 5a). Meanwhile, the transcription levels of *GsERF6* in OX lines were higher than those of its homologs *AtERF5* and *AtERF6* measured by semi-quantitative RT-PCR (Supplementary Fig. S3), indicating that the

**Fig. 4** Temporal expression patterns of *GsERF6* under alkaline stress. **a** Expression levels of *GsERF6* were up-regulated by alkali stress in *G. soja* roots. Total RNA was extracted at the indicated time points from roots of 21-day-old *G. soja* seedlings whose roots were submerged in nutrient solution with 50 mM  $\text{NaHCO}_3$ . Untreated plants were used as controls. Relative transcript levels were determined by qRT-PCR using *GAPDH* as an internal control. The mean value from three fully independent biological repeats and three technical repeats is shown. **b** GUS expression in transgenic *Arabidopsis* under 50 mM  $\text{NaHCO}_3$  treatment. The GUS activity is controlled by *GsERF6* promoter







**Fig. 5** Overexpression of *GsERF6* in *Arabidopsis* enhanced the alkaline tolerance. **a** The transcript levels of *GsERF6* in the WT and OX lines was analyzed by semi-quantitative RT-PCR, the *Actin2* gene was used as an internal standard. **b, c** The growth and germination rate of WT and OX seedlings on 1/2 MS medium with 6 and 8 mM NaHCO<sub>3</sub>. Photographs were taken 12 days after germination. **d** Quantitative evaluation of leaf opening and greening rate at the seed germination stage. Experiments were performed at least three times. The bars represent standard errors. The data show means (±SE) of

three replicates (each with 100 seeds for each line). \**P* < 0.05, \*\**P* < 0.01 by Student's *t* test. **e, f** Phenotypes and measurements of primary root lengths of WT and OX seedlings under normal and alkali stress. Seven-day-old seedlings grown on 1/2 MS were transferred to fresh solid agar plates supplemented with or without 6 mM NaHCO<sub>3</sub>. Photographs were taken after 10 days growth on the supplemented media. All values are means (±SE) from three independent experiments (30 seedlings per experiment). \**P* < 0.05, \*\**P* < 0.01 by Student's *t* test

expression of *GsERF6* is high enough to have an effect on regulating genes in *Arabidopsis*.

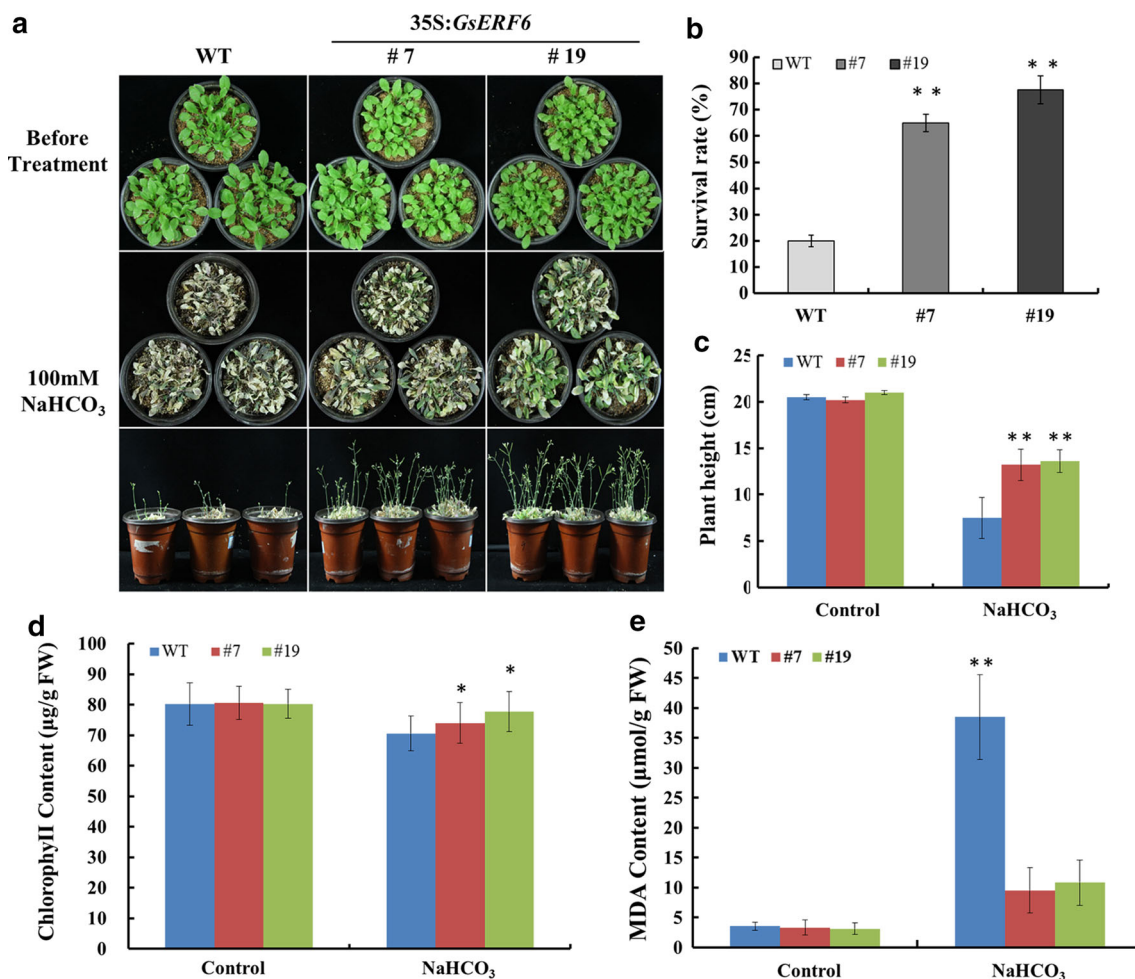
In the absence of NaHCO<sub>3</sub>, *GsERF6* OX plants displayed similar performance with the WT plants, showing no difference in germination and development under

normal conditions (Fig. 5b). In the presence of 6 mM NaHCO<sub>3</sub> (pH 7.25 ± 0.05) or 8 mM NaHCO<sub>3</sub> (7.70 ± 0.05), both the WT and OX lines exhibited very different initial but similar final germination rates up to 100 % (Fig. 5b, c). However, 12 days after germination,

the OX lines had much higher percentages of seedlings with open and green leaves than the WT plants under various concentrations of  $\text{NaHCO}_3$  (Fig. 5b, d). For example, in the presence of 6 mM  $\text{NaHCO}_3$ , the WT seedlings had approximately 30 % individuals with fully open cotyledons while *GsERF6* OX seedlings had approximately 75 %. When  $\text{NaHCO}_3$  concentration increased to 8 mM, the percentage of the WT seedlings with fully open cotyledons was <5 % compared with approximately 20 % for the *GsERF6* OX seedlings (Fig. 5d). We further investigated the effect of alkaline stress on root growth. As shown in Fig. 5e, f, when grown on solid medium supplemented with 6 mM  $\text{NaHCO}_3$ , the leaf growth and root elongation of the WT plants were inhibited more severely than those of OX plants.

During the late growing and developmental stages, 4-weeks old OX lines grown in soils irrigated with

100 mM  $\text{NaHCO}_3$  (pH 8.2) for 16 days showed greater development ability than the WT plants (Fig. 6a). Under normal conditions, the WT plants and OX lines showed similar growth, while OX lines showed significant alkali stress tolerance with higher survival rate and greater height than WT plants after  $\text{NaHCO}_3$  treatment (Fig. 6a–c). Then we measured the total chlorophyll contents in the plants growing in pots after 100 mM  $\text{NaHCO}_3$  treatment. The contents of total chlorophyll were decreased in both WT and transgenic plants, but *GsERF6* OX plants decolor less than the WT (Fig. 6d). These results indicate that *GsERF6* overexpression alleviates the effects of high-alkali stress on chlorophyll deformation and enhances the alkaline tolerance of transgenic plants. It is generally accepted that the level of malondialdehyde (MDA) produced during peroxidation of membrane lipids is often used as an indicator of oxidative damage (Weber et al. 2004; Kotchoni and



**Fig. 6** Enhanced tolerance of transgenic plants to alkaline stress at the adult stage. **a** Phenotypes of WT and OX plants in response to alkali stress. **b** The survival rate of WT and OX plants under alkali stress. **c** The plant height of WT and OX plants. **d** The total chlorophyll content of WT and OX plants. **e** The total malondialdehyde (MDA) content of WT and OX plants. For the alkaline tolerance

at the adult stage, 4-week-old plants were irrigated with 100 mM  $\text{NaHCO}_3$  solution every 4 days for a total of 16 days. Photos were taken on the 16th day after initial alkali treatment. All values are means ( $\pm$ SE) from three independent experiments (40 seedlings per experiment). \* $P$  < 0.05, \*\* $P$  < 0.01 by Student's  $t$  test

Gachomo 2006). Therefore, we measured the MDA contents in the transgenic and WT plants under both alkali stress and normal conditions, and found that MDA level in WT plants was significantly higher than that in *GsERF6* OX lines (Fig. 6e). Together, these data indicate that overexpression of *GsERF6* enhanced  $\text{NaHCO}_3$ -stress tolerance in *Arabidopsis*.

### Overexpression of *GsERF6* specifically increased bicarbonate tolerance but not high pH tolerance

As  $\text{HCO}_3^-$  ion and high pH resulted from  $\text{NaHCO}_3$  solution are the main contributors to alkali stress, we next examined the tolerating function of *GsERF6* in response to  $\text{HCO}_3^-$  ion and/or pure high pH stress.

To explore the role of *GsERF6* on pure high pH stress, we analyzed plant tolerance under a range of pH from 5.8 to 8.2 at germinating and seedling stages. We grew *Arabidopsis* seedlings on the standard medium (pH 5.8) for 7 days and then transferred the seedlings to 1/2MS medium at pH 5.8 (control), or pH 7.5 or pH 8.2 (adjusted by KOH). We can see both *GsERF6* OX and WT had 100 % seed germination rates and similar growing and developmental performances (Supplementary Fig. S4a). The results from vertical growing plants showed that the primary roots elongation of both OX and WT plants were obviously inhibited under high pH stress, but also no significant difference between *GsERF6* OX and WT plants (Supplementary Fig. S4b, c). Our data indicated that *GsERF6* had no contribution to the single high pH stress.

Then we further characterized the function of *GsERF6* under  $\text{HCO}_3^-$  stress. The existence of  $\text{HCO}_3^-$  gives double injuries including bicarbonate ion and high pH stress caused by bicarbonate ion. For  $\text{HCO}_3^-$  stress, here we used  $\text{KHCO}_3$  instead of  $\text{NaCO}_3$  because potassium ion ( $\text{K}^+$ ) is less toxic than sodium ion ( $\text{Na}^+$ ). As shown in Fig. 7, similar with the phenotype under  $\text{NaHCO}_3$  stress conditions, the OX plants had higher survival rate with more open and green leaves than the WT under 6 mM (pH  $7.35 \pm 0.05$ ) and 7 mM  $\text{KHCO}_3$  (pH  $7.65 \pm 0.05$ ) treatments (Fig. 7a, b), and the OX plants were more tolerant with better growth and longer root length at seedling stage (Fig. 7c, d).

### *GsERF6* overexpression altered expression patterns of stress-responsive genes

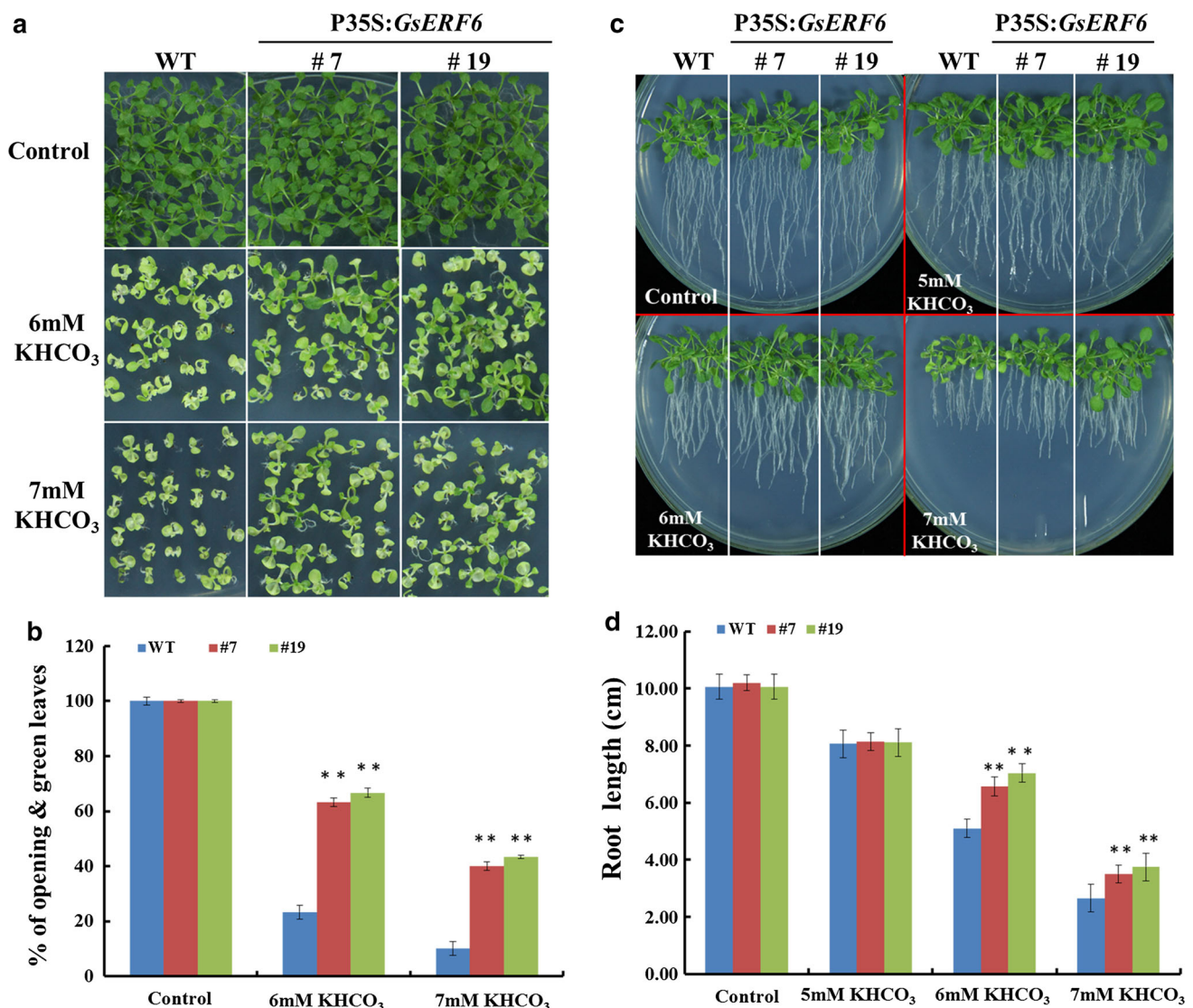
To explore the mechanism of the increased bicarbonate tolerance conferred by *GsERF6* overexpression, the transcription levels of some alkaline stress-related genes were analyzed under 50 mM  $\text{NaHCO}_3$  treatment. Biochemically, NADP-ME,  $\text{H}^+$ -Ppase and  $\text{H}^+$ -ATase have been shown the ability to adjust the pH in the cytoplasm (Marrè and Ballarin-Denti 1985; Kurtz 1987). Therefore we selected

NADP-ME,  $\text{H}^+$ -Ppase and  $\text{H}^+$ -ATase as marker genes under  $\text{NaHCO}_3$  stress. After qRT-PCR analysis, we found that the expression levels of these three genes were significantly induced by  $\text{NaHCO}_3$  stress at 6 and 12 h time points. Both WT and *GsERF6* OX plants showed similar expression profiles (Fig. 8). However, the expression levels were significantly higher in transgenic plants than those in WT at 6 and 12 h (Fig. 8), suggesting that *GsERF6* positively and directly or indirectly regulated the expression of NADP-ME,  $\text{H}^+$ -Ppase and  $\text{H}^+$ -ATase genes. Furthermore, we examined the effects of *GsERF6* on the transcription levels of several other stress-responsive marker genes including *COR47*, *RD29A* and *KINI*. Real-time PCR results showed that in the presence of 50 mM  $\text{NaHCO}_3$  treatment, the expression of all these genes was up-regulated in WT and *GsERF6* OX lines. However, their expression levels were significantly higher in OX lines than WT (Fig. 8). Therefore, we inferred that *GsERF6* might promote the bicarbonate defense by regulating expression of the stress-inducible marker genes.

### *GsERF6* overexpression altered expression patterns of plant hormone synthesis and hormone-responsive genes

Previous studies have suggested that ERF transcription factors can affect the endogenous hormone levels and plant responses to the hormones such as abscisic acid (ABA), ET and JA (Cheng et al. 2013; Lee et al. 2015; Wang et al. 2015; Muller and Munne-Bosch 2015). These hormones are important for plants to adapt to the changeable environments and stress conditions. Therefore, in this study, we further examined the potential role of *GsERF6* to affect the hormone levels and the signal responses by analyzing the expression patterns of some hormone synthesis and responsive marker genes. It is well known that the endogenous ABA levels can be indirectly evaluated by analyzing ABA biosynthesis and catabolism in plant cells (Nambara and Marion-Poll 2005). 9-cis-epoxycarotenoid dioxygenase (NCED) genes encode key enzymes for ABA biosynthesis (Dong et al. 2015). Here, we found that these two ABA biosynthesis-related genes, *AtNCED3* and *AtNCED5*, were both up-regulated under  $\text{NaHCO}_3$ -stress treatment, and their increasing extents in *GsERF6* OX plants are much higher than those in WT plants (Fig. 9). In accordance with this observation, the expression levels of some ABA-responsive genes such as *RAB18* and *RD29B* were also significantly higher in *GsERF6* transgenic plants than in WT even at 12 h after treatment (Fig. 9). We also performed the analyses of ET-synthesis marker genes, *AtACSI*, *AtACS2* and *AtACS5*, and found all were induced under  $\text{NaHCO}_3$  treatment and the increasing fold in *GsERF6* OX plants was much higher than that in





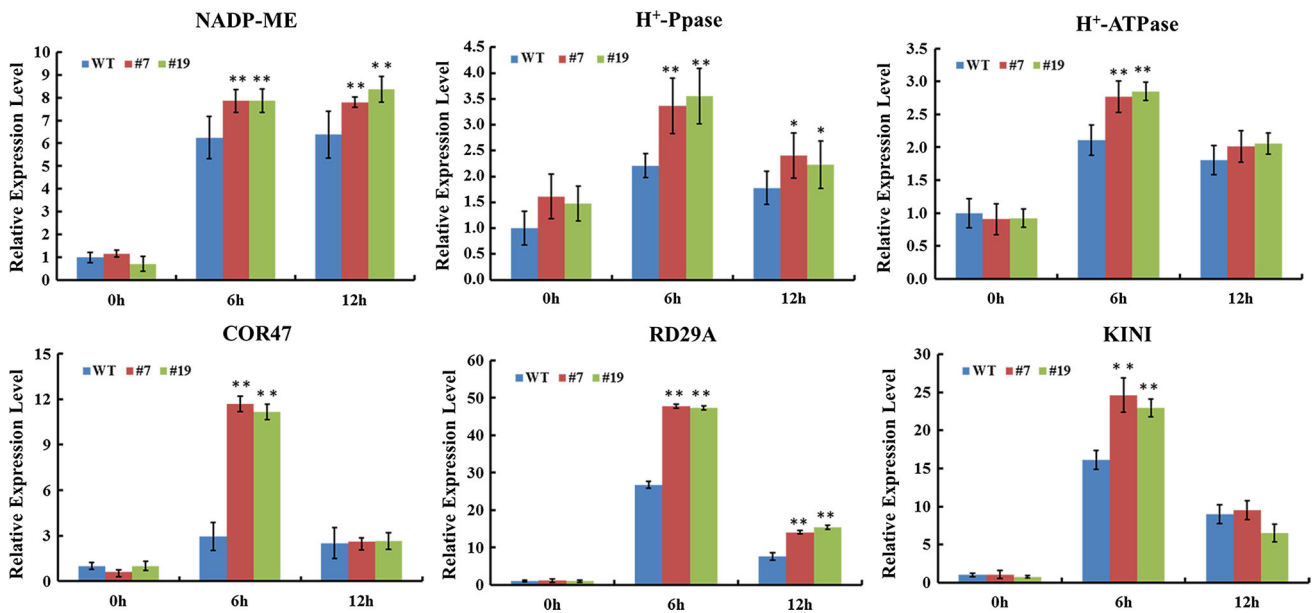
**Fig. 7** Overexpression of *GsERF6* in *Arabidopsis* enhanced  $\text{KHCO}_3$  tolerance. **a** The growth of WT and OX seedlings on 1/2 MS medium with 6 and 7 mM  $\text{KHCO}_3$ . Photographs were taken 12 days after germination. **b** Quantitative evaluation of leaf opening and greening rate. Experiments were performed at least three times. The bars represent standard errors. The data show means ( $\pm$ SE) of three replicates (each with 100 seeds for each line). \* $P < 0.05$ , \*\* $P < 0.01$  by Student's *t* test. **c**, **d** Phenotypes and measurements of primary root

length of WT and OX seedlings under normal and  $\text{KHCO}_3$  stress. Seven-day-old seedlings grown on 1/2 MS were transferred to new solid agar plates supplemented with and without 5, 6 and 7 mM  $\text{KHCO}_3$ . Photographs were taken after 7 days growth on the supplemented media. All values are means ( $\pm$ SE) from three independent experiments (30 seedlings per experiment). \* $P < 0.05$ , \*\* $P < 0.01$  by Student's *t* test

transgenic plants (Fig. 9). The analyses of ET and JA-inducible genes such as *EIN3*, *ERF1* and *PDF1.2* revealed that *EIN3* and *ERF1* were induced under  $\text{NaHCO}_3$  stress. Intriguingly, the expression level of *EIN3* was higher in transgenic plants but the expression level of *ERF1* was lower in transgenic plants than in the WT plants (Fig. 9). In contrast, the expression levels of *ERF1*-target genes such as *P5CS* and *PDF1.2* were not significantly lower in transgenic plants. *P5CS* showed similar expression level compared with WT but *PDF1.2* showed higher expression level in transgenic plants. The similar or higher expression

of *ERF1*-target genes is possibly due to the highly induced levels of hormones such as ABA and JA because *P5CS* can also be induced by ABA and *PDF1.2* can be induced by JA (Cheng et al. 2013). The expression levels of *GsERF6* homologs, *AtERF5* and *AtERF6*, were also examined under  $\text{NaCO}_3$  stress in WT and transgenic plants, and we found both of them were induced under the stress and had higher expression in transgenic plants. Taken together, alkaline ( $\text{NaHCO}_3$ ) stress could induce ABA and ET synthesis and strengthen the hormone responses during adaptation to alkaline ( $\text{NaHCO}_3$ ) stress. The varying expression levels of





**Fig. 8** Expression patterns of stress-induced marker genes in WT and *GsERF6* transgenic *Arabidopsis* seedlings in response to alkali stress. 2-week-old WT and OX seedlings were treated with 1/2 MS solution containing 50 mM NaHCO<sub>3</sub> for 0, 6 and 12 h. The induction of stress-responsive genes *NADP-ME*, *H<sup>+</sup>-ATPase*, *H<sup>+</sup>-Ppase*, *RD29A*,

*COR47* and *KINI* were measured by qRT-PCR analysis. Expression of *Actin2* was used as an internal control. Values represent the means of three biological replicates and three technology replicates for each. \**P* < 0.05, \*\**P* < 0.01 by Student's *t* test

these marker genes are possibly due to the regulation of *GsERF6* across the hormones transduction and feedback, and the further analyses are required to reveal its precise roles in the hormone signaling pathway.

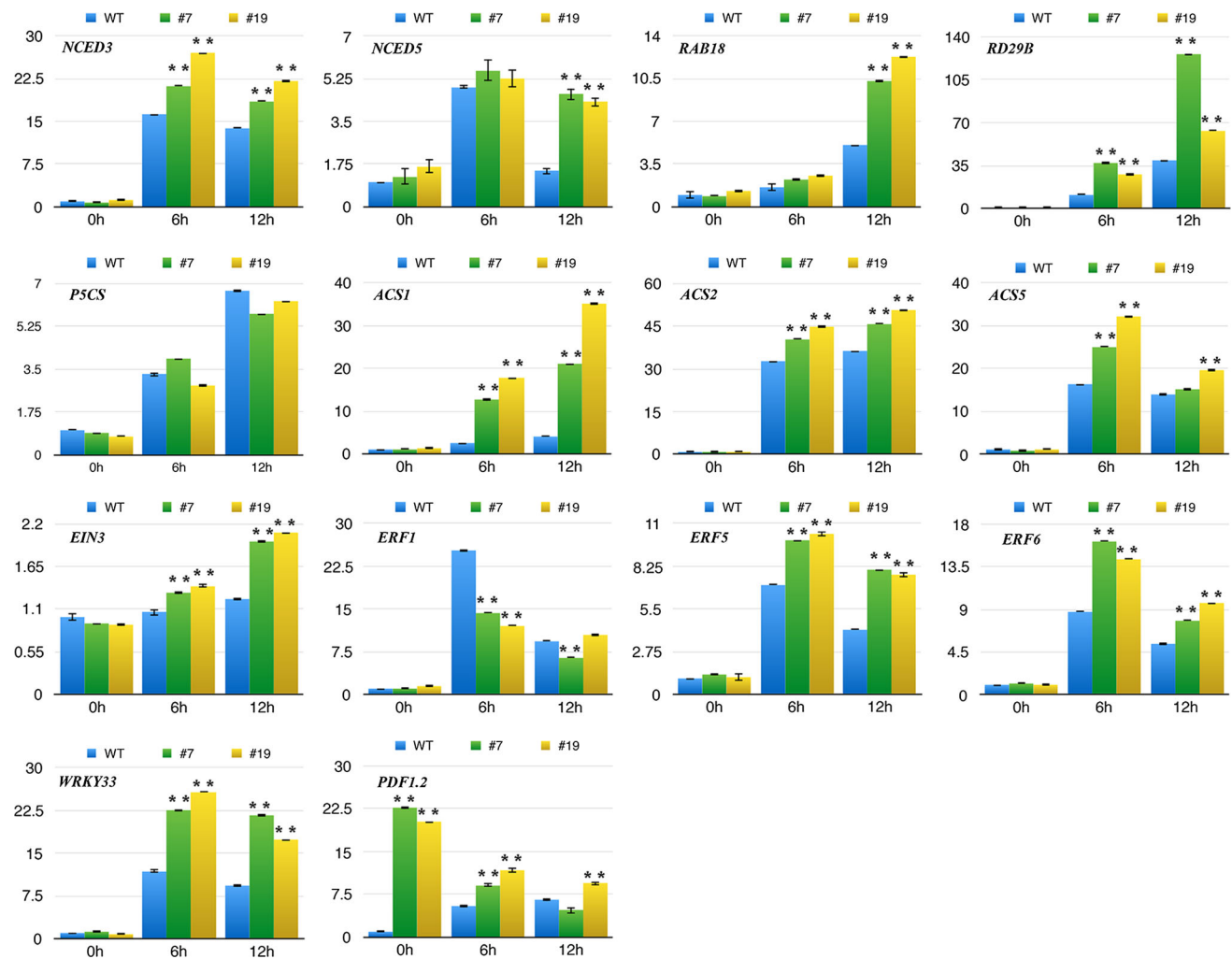
### Discussion

In plants, tolerance or susceptibility to alkaline stress is a coordinated action involved in various genes such as calcium-binding proteins and other components in stress signaling pathways. These components may have cross talk with each other, however little is known on this aspect. Based on the transcriptome sequencing data of wild soybean G07256 roots, 320 TFs belonging to 32 different families were found to be induced during NaHCO<sub>3</sub> treatment (DuanMu et al. 2015). Among them, the ERF TFs were shown to be involved in responses to environmental stresses and exhibited multiple, complex and flexible expression patterns (Xu et al. 2011). In this study, we focused on a novel AP2/ERF family gene *GsERF6*, which was identified as a key gene responding to alkaline stress based on the transcriptome sequencing data.

The AP2/ERF family is a large TF family specifically in plants and the members share a highly-conserved DNA-binding domain (Mizoi et al. 2012). Cluster analysis and phylogenetic tree showed that *GsERF6* was an ERF TF with one conserved AP2 domain and belonged to ERF

superfamily B-3 subgroup (Supplementary Fig. S1). The AP2 domain of *GsERF6* is located in the middle region and shows significant sequence homology with those of the other ERF proteins, but the sequences outside AP2 domain are quite different. Subcellular localization showed that *GsERF6* was a nuclear protein (Fig. 1). Previous studies showed that ERFs usually function as activators or repressors of downstream genes during multiple signals pathways (Shinshi 2008). Our transactivation assays in yeast cells indicate that *GsERF6* had transactivation activity to activated two reporter genes (Fig. 2). These studies suggested that *GsERF6* can putatively function as a TF and have a role in the activation of downstream target genes in plants.

Various TFs show quite different responses to abiotic stresses in plants (Tran et al. 2004; Liu et al. 2007a). Here, we demonstrated that the expression of *GsERF6* displays dynamic changes during NaHCO<sub>3</sub> stress in wild soybean. Consistent with the RNA-seq data, an increase in *GsERF6* expression in *G. soja* roots occurred when the plants were exposed to 50 mM NaHCO<sub>3</sub> (Fig. 4a). This is consistent with the previous findings that other alkali-regulated genes in plants are also regulated at the transcriptional level (Zhu et al. 2011, 2012; Liu et al. 2015). The up-regulation of *GsERF6* during early stages under NaHCO<sub>3</sub> stress may induce downstream components to cope with the alkaline stress. In agreement with the data obtained from the qRT experiment, the GUS activity driven by the *GsERF6* promoter was up-regulated under alkaline conditions in



**Fig. 9** Expression patterns of hormone synthesis and responsive marker genes in WT and *GsERF6* transgenic *Arabidopsis* seedlings under alkali stress. 2-week-old WT and OX seedlings were treated with 1/2 MS solution containing 50 mM  $\text{NaHCO}_3$  for 0, 6 and 12 h. The expression levels of the marker genes were measured by qRT-

PCR analysis. Expression of *Actin2* was used as an internal control. Values represent the means of three biological replicates and three technology replicates for each. \* $P < 0.05$ , \*\* $P < 0.01$  by Student's *t* test

*Arabidopsis* (Fig. 4b). Thus, the *GsERF6* promoter is sufficient to confer up-regulation of *GsERF6*/*GUS* activity under alkaline stress and there may be alkali stress-responsive cis-acting elements in the upstream region of *GsERF6*.

The AP2/ERF genes are also involved in plant growth and development besides stress response. The spatial expression analysis of *GsERF6* in *G. soja* shows it expressed in both the roots and shoots but higher in the roots (Fig. 3a). In *Arabidopsis*, higher expression levels of *GUS* occur in the root stele and in the vascular bundles of leaves and stems (Fig. 3b–l). The tissue-specific expression pattern of *GsERF6* in root stele supports the hypothesis that *GsERF6* plays an important role in the primary uptake and transport of nutrients or ions from the soil. The location of *GsERF6*-*GUS* in stem and leaf vascular bundles proposes a possible role of

*GsERF6* in long-distance transport of nutrients or ions between shoot and root tissues. Based on the specific tissue-location and basic gene expression, we assumed that *GsERF6* may play a direct or indirect role to regulate plant growth and development or alkaline-response.

Some of ERF genes can be induced by the abiotic stresses such as salt, drought and cold, and overexpression of these ERF genes can improve the tolerance to these stresses correspondingly (Xu et al. 2008; Zhu et al. 2010). For example, overexpression of *TaERF1* in *Arabidopsis* improved the salt and cold tolerance (Xu et al. 2007). Overexpression of *RAP2.6L* and *DREB19* also enhanced tolerance to salt and drought stresses (Krishnaswamy et al. 2011). To evaluate the function of *GsERF6* in response to alkaline stress, we generated transgenic *Arabidopsis* with overexpression of *GsERF6* by *Agrobacterium* mediated

transformation. As shown in Figs. 5 and 6, the *GsERF6* OX lines demonstrated an obvious alkali tolerance with higher leaf opening and green leaves rates at the whole plant growing phases, and longer primary roots, higher total chlorophyll content and lower levels of malondialdehyde (MDA) than the wild type. Solaiman et al. (2007) found that canola genotypes maintained higher uptake of P and accumulated greater biomass compared to wheat genotypes on alkaline soils, and the better growth and higher P content in canola genotypes was mainly due to the fact that greater root length could exploit greater soil volume. Thus longer primary roots of *GsERF6* transgenic plants at seedling stage may help the plants to resist against the alkaline stress better (Fig. 5).

Many bicarbonate-regulated genes are involved in metabolism, signal transduction and transcription (Alhendawi et al. 1997). *NADP-MEI*, *NADP-MEII* and *V-H-PPase* have been proven to be induced by bicarbonate stress (Fushimi et al. 1994; Liu et al. 2007b). These proteins play important roles with the function of regulating intracellular pH, helping plant cells to cope with the potential acidification in cytoplasm from environmental stresses (Davies 1986; Tang et al. 1993). In this study, transgenic *Arabidopsis* with *GsERF6* showed increased tolerance to alkaline stress, especially bicarbonate stress, and the expression levels of these detected stress-inducible genes (*NADP-ME*, *H<sup>+</sup>-PPase*, *H<sup>+</sup>-ATPase*, *COR47*, *RD29A* and *KINI*) were significantly higher in *GsERF6* OX lines than in WT plants. The up-regulated expression of other stress-related genes such as *RD29A*, *COR47* and *KINI*, whose transcripts can be strongly induced by cold, drought and ABA (Horvath et al. 1993; Wang et al. 1995; Kurkela and Franck 1990; Seki et al. 2003), may also contribute to the alkaline tolerance in OX lines, because the high accumulation of these proteins plays an important role in adjusting physiological conditions in plant cells (Puhakainen et al. 2004; Msanne et al. 2011). The ability of *GsERF6* upregulating downstream genes suggests that it may be an important regulator in the alkali stress signaling transduction and alkali stress resistance.

Alkali stress, especially alkaline soils in nature, elicits four primary effects on plants: ionic toxicity, osmotic stress, high-pH and bicarbonate. We found that in the 1/2 MS medium, low concentration of  $\text{NaHCO}_3$  (8 mM) resulted in a lethal phenotype but high concentration of salt stress ( $\text{NaCl}$ , 100 mM) did not, indicating that injuries from  $\text{NaHCO}_3$  are mainly due to the high pH and bicarbonate ( $\text{HCO}_3^-$ ) ion, but not to  $\text{Na}^+$ . To elucidate the mechanisms of *GsERF6* transgenic plants responsible for the enhanced alkaline stress tolerance, we analyzed the tolerance of *GsERF6* OX plants under pure high pH stress and  $\text{HCO}_3^-$  ion condition separately. Interestingly, the *GsERF6* OX lines had a significantly increased tolerance to bicarbonate

stress caused by  $\text{NaHCO}_3$  and  $\text{KHCO}_3$ , but showed no tolerance to high pH (Figs. 4, 5, 7; Supplementary Fig. S4). We found that the plants grew better under single alkaline pH treatment than under a similar pH caused by  $\text{HCO}_3^-$  ions (7 mM  $\text{KHCO}_3$  resulting in  $\text{pH } 7.65 \pm 0.05$  in 1/2 MS medium) (Supplementary Fig. S4; Fig. 7). Therefore, we speculate that alkali stress triggers injuries in plants largely through  $\text{HCO}_3^-$  ions, rather than the pure pH, consistent with previous reports (Lee and Woolhouse 1969; Romera et al. 1992; Petersen 1996). The alkali tolerance conferred by *GsERF6* appears to be mainly based on an improved bicarbonate ion tolerance rather than based on pure high pH tolerance. The up-regulated expression of *H<sup>+</sup>-PPase*, *NADP-ME* and *H<sup>+</sup>-ATPase* induced by the pH change in the cytoplasm caused by bicarbonate may help to maintain the homeostasis of the internal environment. Taken together, the novel characteristic of *GsERF6* implies complex roles of AP2/ERF TFs in alkali response; the specific role of *GsERF6* in tolerance to bicarbonate stress provides a perception to elucidate the mechanisms underlying tolerance to alkaline stress.

Plant hormones like ABA, ET and JA are considered to be involved in many aspects of plant growth and development, and responses to environmental challenges including multiple biotic and abiotic stresses (Danquah et al. 2014; Thao et al. 2015). Many ERF subfamily members participate in the stress signaling pathways and serve as connecting factors in the crosstalk of adversity and hormone signaling networks (Pandey et al. 2005; Lee et al. 2015; Wang et al. 2015; Muller and Munne-Bosch 2015). We investigated the effects of *GsERF6* on the hormone synthesis and response in transgenic plants. By performing the qRT-PCR we analyzed the expression pattern of some hormone synthesis and responsive genes in both transgenic and WT plants under  $\text{NaHCO}_3$  stress. The results indicated that  $\text{NaHCO}_3$  stress can increase the expression levels of both ABA and ET synthesis and responsive genes in plants, such as the ABA synthesis genes *AtNCED3* and *AtNCED5*, and the ABA-responsive genes *RAB18* and *RD29B*, ET-synthesis related genes *AtACS1*, *AtACS2* and *AtACS5*, and the ET responsive genes *EIN3*, *ERF1*, *ERF5*, *ERF6* and the JA responsive gene *PDF1.2* (Fig. 9), suggesting that *GsERF6* may regulate plant responses to  $\text{NaHCO}_3$  stress through regulating the synthesis, signaling transduction of the relevant endogenous hormones like ABA, ET and JA. Based on the above findings, we proposed that overexpression of *GsERF6* increases bicarbonate stress tolerance might be due to induction of the plant hormones such as ABA and ET as signaling molecules to activate a number of hormone- and stress-responsive genes such as *RAB18*, *RD29A*, *RD29B* and *COR47* genes, and some ERF like proteins. Consequently, these signaling molecules regulate the defense responses of plants through the synergistic or

antagonistic effect. The mechanisms of GsERF6 and its role in bicarbonate signaling and signal transduction of other plant hormones need to be further analyzed in details.

In conclusion, we demonstrate here a novel TF gene from wild soybean, *GsERF6*, which can modify plant tolerance to alkali stress by specifically increasing tolerance to bicarbonate stress. It is significant to further investigate the precise function and the mechanism of GsERF6 in bicarbonate uptake, translocation and stress resistance. Further investigations include the relationship between bicarbonate stress and the ethylene signal transduction pathway, as well as the role of GsERF6 in the crosstalk of ethylene and bicarbonate-mediated signaling systems are also need to be conducted.

**Author contribution statement** YY and YZ designed the research; YY, AL, XD, SW, CC, LC, JX and QL performed experiments; XS and HD analyzed data; YY wrote the manuscript; DZ and XD revised the manuscript. All authors contributed to editing and approving the final version of the manuscript.

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