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A New AP2/ERF Transcription Factor from the Oil Plant *Jatropha curcas* Confers Salt and Drought Tolerance to Transgenic Tobacco

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Abstract *Jatropha curcas* L. is a drought and salt-tolerant oil plant widely used for various purposes and has considerable potential as a diesel/kerosene substitute or extender. Understanding the molecular mechanisms underlie that the response to various biotic and abiotic stresses of this plant could be important to crop improvement efforts. Here, a new AP2/ERF-type transcription factor gene, named *JcERF2*, was isolated from the leaves of *J. curcas*. Sequence analysis showed that the *JcERF2* gene contains a 759-bp open reading frame encoding a polypeptide of 252 amino acids. The predicted *JcERF2* protein contained a conserved DNA-binding domain (the AP2/ERF domain) with 58 amino acids. The JcERF2 protein is highly homologous with other ERFs. JcERF2 was localized in the nucleus by analysis with a *JcERF2*-green fluorescent protein (GFP) fusion protein. Quantitative polymerase chain reaction (qPCR) analysis showed that *JcERF2* was induced by drought, salt, abscisic acid, and ethylene. Overexpression of *JcERF2* in transgenic tobacco plants enhanced the expression of biotic and abiotic stress-related genes, increased the accumulation of free proline and soluble carbohydrates, and conferred tolerance to drought and salt stresses compared to the wild type (WT). Taken together, the *JcERF2* gene is a novel AP2/ERF transcription factor involved in plant response to environmental factors, which can be used as a potential candidate gene for genetic engineering of crops.

Novelty Statement • A new AP2/ERF-type transcription factor gene, JcERF2, was isolated from the leaves of *Jatropha curcas*.
• Experiments using a binary expression vector, pBI121-JcERF2, in tobacco suggested that JcERF2 plays a role in plant defense reactions.
• The overexpression of JcERF2 in tobacco enhanced the expression of some biotic and abiotic stress-related genes, increased the accumulation of free proline and soluble carbohydrates, and conferred tolerance to drought and salt.

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Keywords Ethylene response factor · Defense reaction · Binary expression vector · Drought tolerance · *Jatropha curcas* · AP2/ERF · Transgenic

Introduction

Plant growth and productivity are greatly limited by environmental stresses, of which drought, high salinity, and low temperature are the most serious threats in many areas of the world. Being sessile in nature, plants have evolved protective mechanisms to resist unfavorable conditions [8]. The protection is manifested by genes to modulate specific transcription factors via transduction cascades and transcriptional activation of series defense genes [45]. The AP2/ERF transcription family is a large family of plant transcription factors that includes four subfamilies called AP2, CBF/DREB, ERF, and RAV according to their types and numbers of DNA-binding domains [41]. The AP2 subfamily consists of two AP2/ERF domains that are mainly involved in the regulation of plant development [4, 9, 12]. The RAV subfamily, containing an AP2/ERF domain and a B3 domain, differs from the other transcription factor subfamilies, and some RAV transcription factors may cause transcriptional inhibition [23]. The CBF/DREB and ERF subfamily proteins contain one AP2/ERF domain each. The genes in the CBF/DREB subfamily primarily regulate the responses of plants to abiotic stress by recognizing the dehydration-responsive element or C-repeat (DRE/CRT). In contrast, the ERF subfamily regulates the responses of plants to biotic stress by recognizing the GCC box. Hundreds of transcription factor genes have been cloned from various plants since the first transcription factor was cloned from *Zea mays* by Paz-Ares [39].

The plant-specific ERF transcription factor family has numerous members among land plants and is involved in the signal transduction of the response to drought, salt, disease, and other biotic and abiotic stresses [6, 34]. The AP2/ERF transcription factor *APETALA2* (AP2) gene was first isolated by Jofuku et al. [25] from *Arabidopsis*, after which the ERF transcription factors were successively isolated and studied in tomatoes [17], *Arabidopsis thaliana* [35], *Nicotiana tabacum* [13], pepper [49, 53], wheat [11], barley [26], rice [32], soybean [3, 7], cotton [24] and peanuts (*Arachis hypogaea L.*).

In rice, Cao et al. [5] showed that *OsBIERF1*, *OsBIERF3*, and *OsBIERF4* were upregulated by salt, cold, drought, and wounding. The transcripts of *TERF1* significantly accumulated under drought, cold, and abscisic acid (ABA) stress and involved in the ethylene and osmotic regulation pathways in tomato [56]. Overexpression of *GmERF3* in tobacco increased their tolerance to salt, drought, and diseases [54]. Similarly, overexpression of *RsERF1* in *Arabidopsis* exhibits improved expression of abiotic stress-related genes (e.g., *ABF3*, *ABF4*, *ADH*, *Rab18*, *SUS1*) and enhanced resistance to drought [3]. Studies have demonstated that ERF can increase tolerance to salt [3], drought [54], cold [53], high temperature, heavy metals, pathogens [49], and other biotic and abiotic stresses by activating or inhibiting expression of the target gene in ethylene signal transduction [18, 34]. ERF proteins may play pivotal roles in plant signal transduction and may be associated with different pathways [51]. Thus, the molecular mechanisms of ERF transcription factors should be investigated completely to contribute to agriculture and production.

Jatropha curcas L., belonging to the family of Euphorbiaceae, is a perennial, deciduous, drought-resistant shrub tree. It is native to Central America and widely distributed in tropical, subtropical, and hot, dry, valley areas, especially in Central and South America, Africa, India, and Southeast Asia [43]. *J. curcas* can be used to make biodiesel and biopesticides, in medical

applications, to conserve soil, form biological hedges, provide animal feed, and other purposes [36]. The value of *J. curcas* as a biodiesel energy which may replace petroleum energy is receiving more attention because the depletion of traditional fossil fuels. As the genome of Jatropha being sequenced [21, 42] and genetic transformation technology being established [27, 29, 30, 37], the identification of a large number of agronomically important genes is now in progress. Nevertheless, few studies have focused on transcription factor genes in *J. curcas*. Only two papers have reported *J. curcas* AP2/EREBP transcription factors until now [47, 48].

In the present investigation, we cloned a new *J. curcas* ERF gene (*JcERF2)* by RACE PCR. Bioinformatics and expression analyses reveal that the predicted protein was located in the nucleus and induced by drought, salt, abscisic acid, and ethylene. Expression analysis was performed under stress conditions. Overexpression of *JcERF2* transgenic tobacco plants was constructed to fully illustrated the molecular mechanism of *JcERF2.*

Materials and Methods

Plant Materials and Treatments

J. curcas seeds were collected from Xichang, Sichuan Province, China; germinated in moist vermiculite; and transplanted to a 1:1 mixture of nutrient soil and vermiculite under a 16-h light/8-h dark photoperiod at 28 °C. Seedlings grown to the five to six true-leaf stages were exposed to salt, drought, ABA, or ethylene. For salt and drought stress treatments, seedling roots were washed and immersed in 0.3 M NaCl and 20 % PEG6000, respectively [47]. For the ABA treatment, seedlings were sprayed with 200 μM ABA dissolved in 0.01 % alcohol solution [54]. Ethylene treatment was performed with 2 mL 40 % ethephon and 1 g $NaHCO_3$ dissolved in 200 mL H_2O [55]. To test the expression pattern of the *JcERF2* gene, the treated materials were sampled at 0, 1, 3, 6, 12, 24, and 48 h, respectively, then frozen in liquid nitrogen and kept at −80 °C for further analysis.

Tobacco leaves were subjected to 70 % ethanol for 30 s, washed in sterile water three times, then disinfected in 0.1 % mercuric chloride for 8 min, and finally rinsed several time with sterile distilled water. The cotyledons were taken out from the seeds and placed in 40 mL Murashige and Skoog (MS) medium (Murashige and Skoog [33]) with 0.6 % (*w*/*v*) agar, pH 5.8, placed in the dark for 3 days, and then incubated at 26 °C in a 16-h light/8-h dark photoperiod.

Isolation and Sequence Analysis of the *JcERF2* Gene

Total RNA was prepared from young leaves of *J. curcas*, using an RNA extraction kit (Tiangen Biotech, Beijing). Poly $(A)^+$ RNA was isolated using an mRNA purification kit (Amersham Pharmacia Biotech, USA). The molecular cloning of the *JcERF2* gene was accomplished by synthesizing first-strand complementary DNA (cDNA) from total RNA using a Primescrip RT reagent (TaKaRa, Dalian, China) and storing it at −20 °C. A 143-bp cDNA fragment was amplified from cDNA using AP2/ERF conserved domain degenerated primers (5′TGGG G(A/G)AAAT(T/G)(G/C)GC(T/G)GC3′ and 5′AA(A/G)TT(C/A/G)AC(T/C)TTAGCTTT3′). Degenerated primers were designed using the conserved sequence TRGV RQRPWGKVAEIRDP, commonly present in most of the ERF family.

The 143-bp cDNA fragment was cloned into pMD19-T easy vector for sequencing, and was confirmed to be an ERF gene. Next, 5′-RACE/3′-RACE methods were employed to extend the fragment into a full-length cDNA gene. The cDNA was obtained, denoted as *JcERF2*, and deposited in GenBank with the accession number of JF518881.1. The gene sequence of *JcERF2* was analyzed by DNAMAN software, MEGA.5.1 software, a database search using Blastx (http://www.ncbi.nlm.nih.gov/), and the isoelectric point and molecular weight of *JcERF2* were predicted by ExPASy (http://www.expasy.org/).

Expression of *JcERF2* in Response to Stress Treatments

After selecting five to six true leaves from similarly sized seedlings, we exposed the seedlings to salt, drought, ABA, and ethylene and then sampled leaves at 0, 1, 3, 6, 12, 24, and 48 h. The expression of *JcERF2* under these stress conditions was detected using real-time quantitative polymerase chain reaction (qPCR), and the *18sRNA* gene of *J. curcas* was used as the reference gene. The primers were 5′ AGGAATTGACGGAAGGGCA3′ and 5′ GTGCGG CCCAGAACATCTAAG3′ for *18sRNA* and 5′TGCTTCTGCCCCTTCTCG3′ and 5′TTGTTG GTGCTGCTGTGATG3′ for *JcERF2*.

Subcellular Localization Analysis of *JcERF2*

We constructed a transient expression vector by fusing *JcERF2* to the N-terminal of green fluorescent protein (mGFP) under the control of CaMV 35S promoter (pBI221-*JcERF2*-mGFP). The recombinant vector was transferred into *Arabidopsis* protoplasts that were isolated from leaves as described by Abel and Theologis [1]. We cultured these for 24 h in the dark at 25 °C and then observed them using a confocal scanning laser microscope (Leica Microsystems, Heidelberg, Germany).

Generation of Transgenic Tobacco Plants

To produce overexpression tobacco, we amplified the open reading frame of *JcERF2* using PCR. The product (759 bp) was cloned into the binary expression vector pBI121 (Invitrogen, USA) under the control of the CaMV 35S promoter. The transformation of tobacco was performed by the floral dipping method (Clough and Bent [10]) using *Agrobacterium tumefaciens* strain LBA4404. The transgenic plants were obtained through the Kan resistance screening of the pBI121 vector. Positive plants were obtained by RT-PCR analysis using total RNA that was extracted from transgenic tobacco as the template. T2 seeds of transgenic tobacco plants were harvested for use in later experiments.

Germination Experiment of *JcERF2* Transgenic Tobacco

The seeds of transgenic and wild tobacco were sterilized as described previously and sown in ½ MS medium with different concentrations of NaCl (0, 50, 100, 150, 200 mM/L). They were maintained at 26 °C in the dark for 3 days and then 26 °C, in a 16-h light/8-h dark photoperiod for 7 days. We determined the germination rate and the root lengths of seedlings, each using 50 seeds, with three independent experiments. The data were analyzed with SPSS, version 13.0.

Tolerance of Overexpression Tobacco to Drought

The transgenic and wild tobacco seedlings were grown to the five to six true-leaf stages and treated with 30 % PEG600 for 15 days. We observed the phenotypic changes and detected the amounts of free proline [44], soluble carbohydrate [50], of the sample leaves from the control and stress treatment.

Expression Analysis of Putative Downstream Genes of *JcERF2*

Five putative downstream genes of *JcERF2* were selected for analysis: *NtPR1a* (X06930.1), *NtOsmotin* (M29279.1), *NtSAR8.2a* (M97194.1), *NtP5CS* (HM854026.1), and *NtSPSA* (AF194022.1). Tobacco *actin* gene (JQ256516.1) was used as an internal control, and we explored the expression of these genes in wild and transgenic tobacco using quantitative real-time PCR. The primers that we used are shown in Table 1. Three replicates were performed.

Results

Molecular Cloning and Sequence Analysis of *JcERF2*

To understand the role of the ERF transcription factors from *J. curcas*, the novel gene *JcERF2* was cloned from *J. curcas* leaves with degenerate primers based on the conserved amino acid domains of ERFs from other plants by RACE technology. Sequence analysis revealed that the full length of *JcERF2* was 1367 bp, with an open reading frame (ORF) of 759 bp, a 5′-untranslated region (UTR) of 274 bp, a 3′UTR of 334 bp, and encodes a protein of 252 amino acids. According to ExPASy (http://web.expasy.org/), we predicted that the molecular weight of *JcERF2* was 28.3 kDa with a isoelectric point (pI) of 5.83. The predicted JcERF2 protein has only one typical AP2 DNA-binding domain between 56 and 113 amino acid residues, suggesting that *JcERF2* belongs to the ERF subfamily of the AP2/ERF family [19].

Table 1 Primers used for quantitative real-time polymerase chain reaction analysis

Primer names	Primer sequences (5′-3′)
NtActin-F	GACAATGGAACAGGAATGGTCAAGGC
NtActin-R	CCAGTTGCTGACAATTCCATGCTC
JcERF2-F	GGTTTCAGCACTCTCTCAAGTCATCGC
JcERF2-R	GGTCTTTGCCTCACTCCTCTGTAG
NtOsmotin-F	GTAACTTCAATGCTGCTGGTAGG
NtOsmotin-R	GTCCAAAGCGTATTCAGCCAAGGTG
NtP5CS-F	GAGACAGTGATTACTTTTGGAGAC
NtP5CS-R	GCCAGCATAAGCAGCATACATAGCAGC
NtPR1a-F	CTCAACAAGACTATTTGGATGCC
NtPR1a-R	CCTCAGCTAGGTTTTCGCCGTATTG
NtSAR8.2a-F	GCTGGTGTGAGTCTAGTTTTGAGG
NtSAR8.2a-R	GACCCAAGACTTGTTGCAAGTTCC
NtSPSA-F	GCAGAGAGGCAGTTGCTGATATGTC
NtSPSA-R	CCAGGCTTCCATTGTCTCCAC

Subcellular localization signal analysis was conducted by http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_y.cgi, and it indicated that there was no nuclear localization signal in this protein.

Homology Analysis of *JcERF2* Amino Acid Sequences

Blastx (http://www.ncbi.nlm.nih.gov/) analysis showed a high similarity between the predicted *JcERF2* protein and other plant ERF proteins. Notably, *JcERF2* shared a highest degree of similarity with *Ricinus communis* (80 %, XP002512669.1), as both *J. curcas* and *R. communis* are members of the family Euphorbiaceae. Homologous comparison also displayed that *JcERF2* shared different degree of similarity with other ERFs from *Morus notabilis* (78 %, EXB64474.1), *Cucumis sativus* (78 %, XP004142046.1), *Arabidopsis lyrata* (74 %, XP002881295.1), *Glycine max* (74 %, XP003522452.1), and *Citrus clementine* (73 %, XP006436961.1) (Fig. 1a), which suggests that *JcERF2* is a new member of AP2/ERF gene family. In order to investigate the evolutionary position of *JcERF2* among the phylogenetic tree of various ERFs, a phylogenetic tree of ERFs was constructed using MEGA 5.1 software as shown in Fig. 1b. *JcERF2* showed the closest relationship with *R. communis*.

Expression Pattern of *JcERF2* Gene Under Different Stress Conditions

As *J. curcas* can survive with high levels of salinity and dehydration, we have tested the expression level of *JcERF2* in response to 0.3 M NaCl and 20 % PEG6000 in different time points. We also investigated the *JcERF2* expression level when the seedlings were treated with ABA and ethylene since ERFs are involved in plant hormone signal transduction. The expression levels of *JcERF2* were low under normal conditions but increased in response to salt, drought, ABA, and ethylene.

JcERF2 gene showed the highest level when exposed to 0.3 M NaCl for 1 h which indicates the irritability of plants, but in 20 % PEG6000, it appeared the highest level after a period time of 12 h stress, which may suggest that NaCl is a fast stimulator for *JcERF2* without considering about the concentration of NaCl and PEG6000. When the seedlings were handled with ABA and ethylene, *JcERF2* exhibit the highest expression level at 24 and 48 h. The expression level of *JcERF2* first rise and then drop with the increasing of treatment duration. These phenomena may be explained by the irritability and adaptation of plant response to stress. Different stimulates caused by different expression level, from our data (Fig. 2), of the four treatments PEG6000 can lead to the highest expression level of *JcERF2* much more than ABA, and followed by ethylene and NaCl. Thus, we can draw the conclusion that drought prompted the greatest increase expression of *JcERF2*. Further study still needed to explain the exact mechanism because our sampling time was not enough.

Subcellular Localization Analysis of JcERF2 Protein

Subcellular localization of proteins is important to elucidate their function in cells. Since *JcERF2* is a transcription factor, it is expected to function in the nucleus, but bioinformatic analysis did not reveal a nuclear localization signal. Therefore, an in vivo targeting experiment was performed to fuse the JcERF2 coding region to

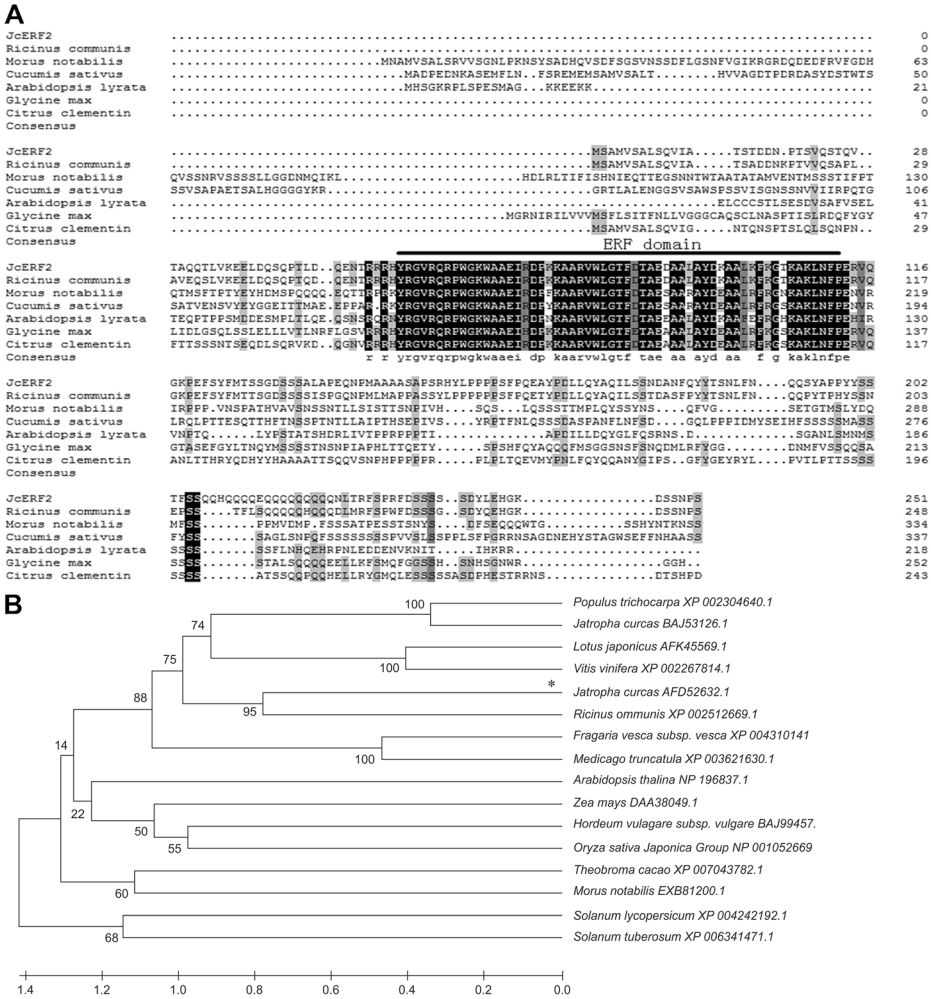

Fig. 1 Multiple sequence alignment and phylogenetic analysis of JcERF2. **a** Comparison of deduced amino acid sequences of AP2/ERF-related proteins that have high sequence similarity with JcERF2: *Ricinus communis* (XP002512669.1), *Morus notabilis* (EXB64474.1), *Cucumis sativus* (XP004142046.1), *Arabidopsis lyrata* (XP002881295.1), *Glycine max* (XP003522452.1), and *Citrus clementina* (XP006436961.1). Amino acid residues that are conserved in seven proteins are shown in *dark gray*. The sequence of *JcERF2* between 56 and 113 bits is a conserved DNA-binding domain (ERF domain) marked by a transverse line. **b** The phylogenetic tree of JcERF2, showing the relationship between JcERF2 and other ERFs. It was generated using the unweighted pair-group method with arithmetic means (UPGMA) algorithm by MEGA4.1 software. The *scale bar* represents genetic distance

the N-terminus of the mGFP gene under the control of the CaMV 35S promoter, transient expression vector transduction into *Arabidopsis* protoplasts [1]. Empty vector was used as a control. Localization of the fusion protein was then detected with a confocal laser fluorescence microscopy. As shown in Fig. 3, the JcERF2/mGFP fusion protein was exclusively located in the nucleus, whereas the control mGFP was uniformly distributed throughout the cell.

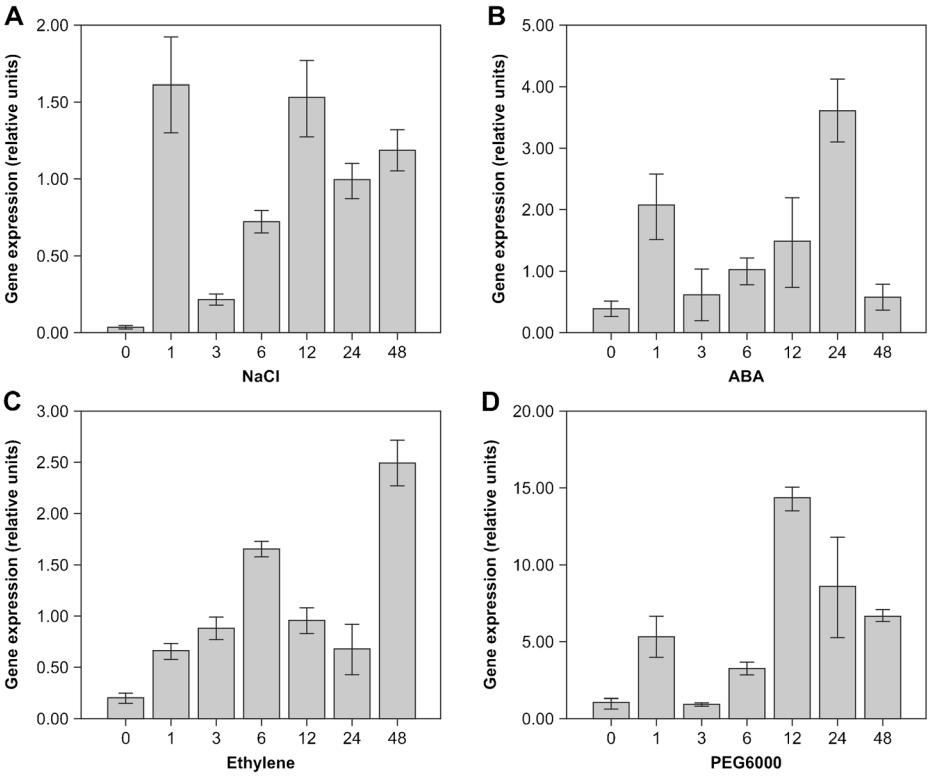

Fig. 2 Expression of the *JcERF2* gene in response to different stresses. Samples were taken at 0, 1, 3, 6, 12, 24, and 48 h. The expression of *JcERF2* was detected by real-time qPCR, and the *18sRNA* gene of *Jatropha* was used as the reference gene. **a** 300 mM NaCl, **b** 100 mM abscisic acid (ABA), **c** 20 % ethylene, **d** 30 % PEG6000. The mean value and standard deviation were obtained from three independent experiments

Overexpression of *JcERF2* Enhanced Expression of Stress-Related Genes in Tobacco

In order to further study the function of *JcERF2*, we constructed the overexpression vector pBI121-*JcERF2*, in which *JcERF2* was under the control of the CaMV 35S promoter. The vector was transformed into wild-type tobacco by the *Agrobacterium*-mediated leaf disc transformation method, seeds of the T2 generation harvested. Studies have demonstrated that PRs, osmotin, and SAR8.2 are GCC-box containing genes, their expression can be modulated by different ERF transcription factors [13, 17, 38]. According to the previous reports, P5CS mainly induced under salt, drought, ABA, and P5CS2 has a constitutive housekeeping gene [2]. So, we detected the expression levels of *JcERF2*, *NtOsmotin*, *NtPR1a*, *NtSAR8.2a*, *NtP5CS*, and *NtSPSA* by quantitative real-time PCR. As shown in Fig. 4, there was no expression of *JcERF2* gene in wild-type tobacco, while in transgenic tobaccos, the expression of *JcERF2* was significantly high. The expression of *JcERF2* in transgenic tobacco implied that it can function well in tobacco, which suggests that *JcERF2* can also express in other crops. The expression level of *NtOsmotin*, *NtP5CS*, *NtPR1a*, and *NtSAR8.2a* in transgenic tobacco is all higher than that in wild type ($p<0.05$). But, the expression level of *NtSPSA* increased not so much as the others since the wild type also has a higher level of expression.

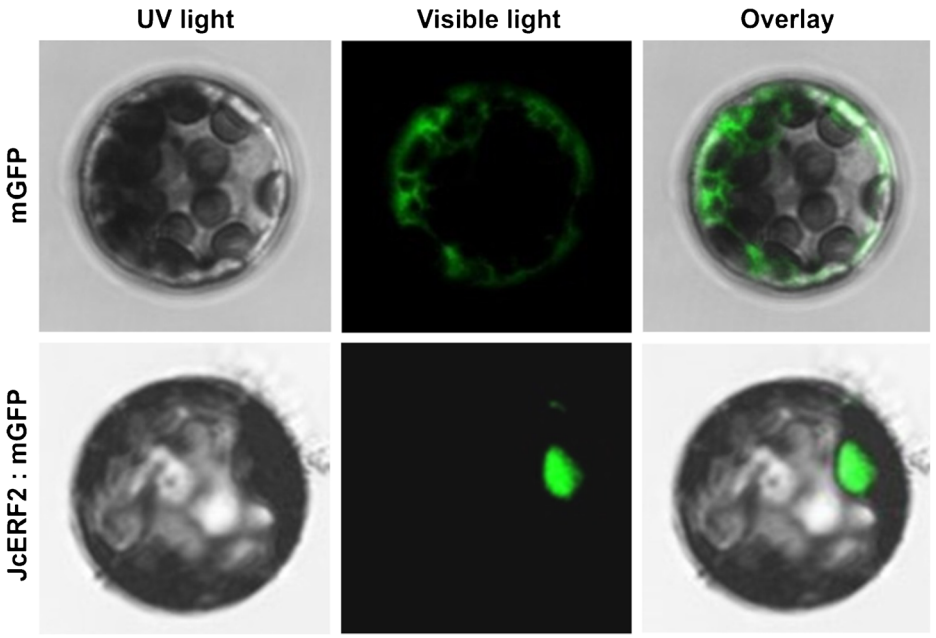

Fig. 3 Subcellular localization of JcERF2 protein in *Arabidopsis* protoplasts. *Arabidopsis* protoplasts were transiently transformed with constructs containing either a control mGFP or JcERF2/mGFP under the control of the CaMV35S promoter. The subcellular location of the JcERF2/mGFP fusion protein or control mGFP alone were viewed with a confocal scanning laser microscope 24 h after transformation. Bright field images (*left*), fluorescence images (*middle*), and the corresponding overlay images (*right*) of representative cells expressing mGFP or JcERF2/mGFP fusion protein are shown

The five selected gene showed a similarly expression level in the transgenic tobaccos suggested that those genes were modulated by the ERF subfamily and *JcERF2* can regulate many genes involved in stress response.

Seed Germination Assays

In order to determine the resistance of transgenic tobacco to salt, we conducted a germination experiment using the seeds of wild and transgenic tobacco. The seeds were sown in ½ MS medium containing different concentrations of NaCl. We determined the germination rate and root length of these seedlings. When treated with NaCl, the germination rate was significantly different; even at a low level of 50 mM NaCl stress, the transgenic tobacco seeds showed a higher rate of germination than wild type, and all of them showed a decrease germination rate with the increase of the NaCl concreation ($p<0.01$) (Fig. 5a, b). The data also showed that few of the wild-type seeds can survive with 200 mM NaCl, and the germination ones showed growth retardation when compared with the transgenic tobacco seeds. The root length of transgenic and wild-type tobacco seedlings exhibits the same tendency, as shown in Fig. 5c, d. The wild-type and transgenic tobacco seedlings reached the same root length of 1.25 cm without NaCl, but the wild-type seedlings showed a more decrease in root length than the overexpression seedlings when the concentration of NaCl is

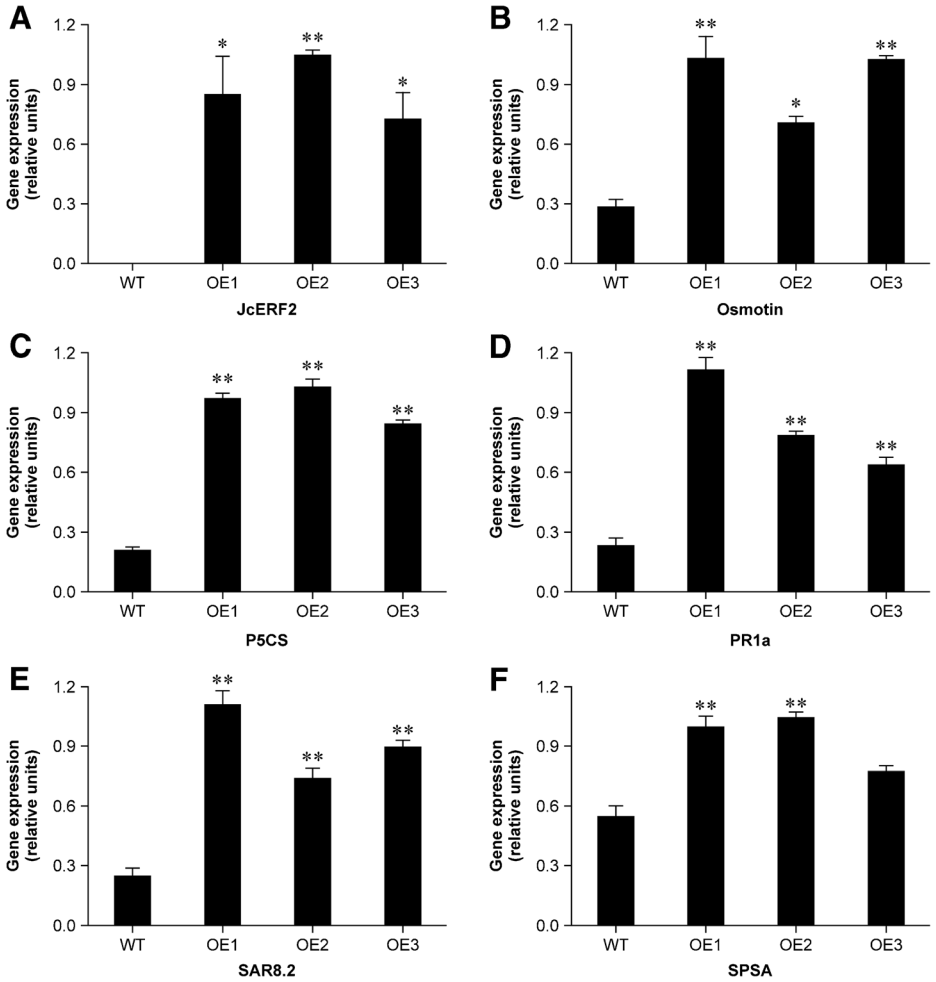

Fig. 4 Expression analysis of *JcERF2* and putative downstream genes in wild-type and transgenic tobacco under normal conditions. Total RNA was isolated from the plants and analyzed by RT-qPCR using actin gene as a template control. *OE1* to *OE3* indicate *JcERF2*-transformed independent tobacco lines. The putative downstream genes are NtOsmotin (M29279.1), NtP5CS (HM854026.1), NtPR1a (X06930.1), NtSAR8.2a (M97194.1), and NtSPSA (AF194022.1). *Error bars* indicate SD from three independent experiments (*$p<0.05$, **$p<0.01$)

increased. The length of wild-type seedlings is less than 1 cm with a 50 mM NaCl; the transgenic tobacco seedlings emerge this phenomenon when the NaCl concentration reached as much as 150 mM ($p<0.01$) (Fig. 5c, d). These results demonstrated that overexpression of *JcERF2* in tobacco improved seed germination rate and tolerance to NaCl.

Defense Response is Enhanced in Transgenic Tobacco to Drought Stress

Soluble sugar and free proline are important physiological indicators of stress tolerance; these substances are positively correlated with response to stress in plants. In order to explore the

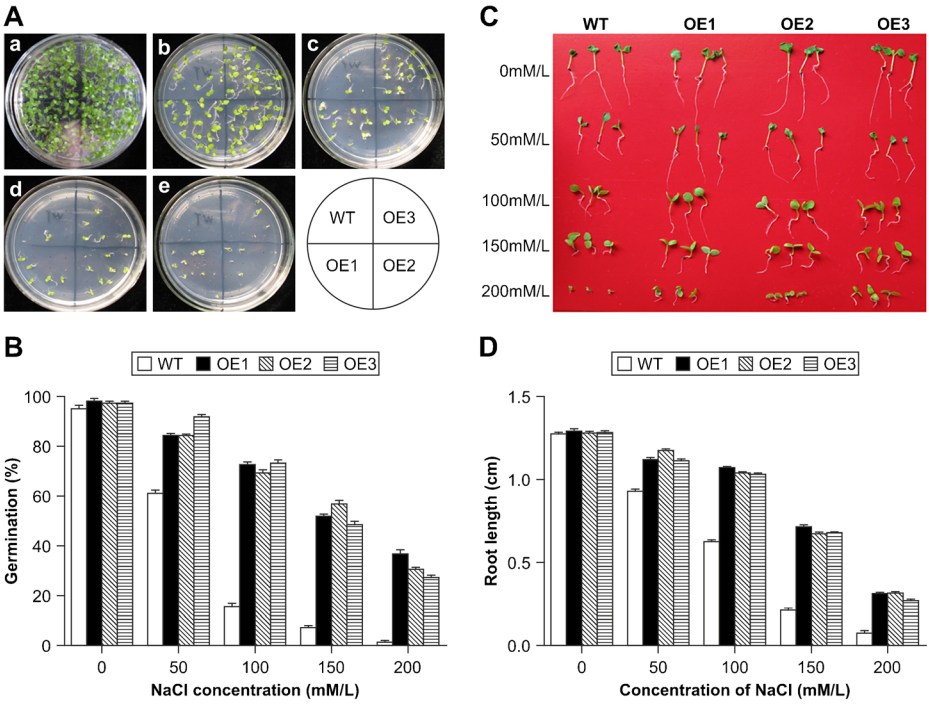

Fig. 5 Effect of salt stress on the seeding stage of wild-type (WT) and transgenic (*OE1–OE3*) tobaccos. **a** Tobacco seedlings grown for 7 days in Murashige and Skoog medium containing *a* 0 mM NaCl, *b* 50 mM NaCl, *c* 100 mM NaCl, *d* 150 mM NaCl, or *e* 200 mM NaCl. **b** Tobacco seedlings germination rate under salt stress. (Results are presented as mean±SD from three independent experiments, and 50 seeds of each line were grown for each experiment.) **c** Tobacco seedlings grown for 7 days in Murashige and Skoog medium containing 0, 50, 100, 150, or 200 mM NaCl. **d** The root length of tobacco seedlings under salt stress. (Results are presented as mean±SD from three independent experiments, and 20 seeds of each line were grown for each experiment.) The differences between the transgenic line and the WT plants are significant ($p<0.01$) when salt stress is present

functions of the *JcERF2* gene in transgenic tobacco defensive reactions, seedlings of transgenic and wild-type tobaccos were irrigated with 30 % PEG6000 and leaves were collected after 15 days. The wild-type tobaccos were wilted slowly during the treatment, while the transgenic tobacco remain healthy and grow well (Fig. 6a).

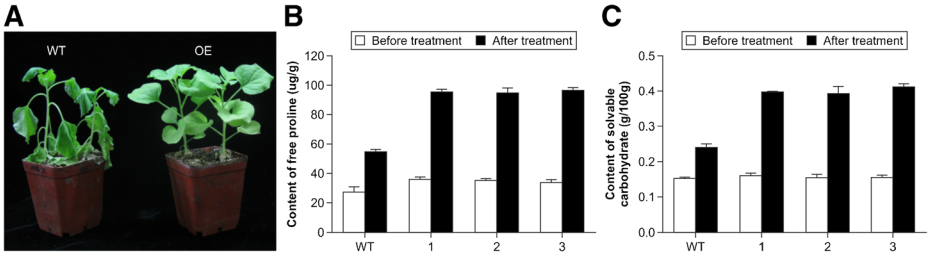

Fig. 6 Effects of drought stress on wild-type and transgenic tobacco plants. **a** The phenotypic changes in wild-type and transgenic plants 15 days after treatment with 30 % PEG, *WT* wild-type tobacco, *OE* transgenic tobacco. **b** Effects of drought stress on amounts of soluble carbohydrate. **c** Effects of drought stress on amounts of free proline. *WT* wild type, *1–3* different transgenic tobacco plants. Results are presented as mean±SD from three independent experiments

Analysis of the amounts of free proline and soluble carbohydrates of the samples were performed. The wild-type and transgenic seedlings showed the same amounts of soluble carbohydrates in leaves before treatment, but the proline concentration is higher in transgenic tobacco seedlings than wild type (Fig. 6b, c), which implied that *JcERF2* modulate the expression of the two substances by different mechanism. All the seedlings showed a higher concentration of proline and soluble carbohydrates when exposed to PEG6000, but the increase is significantly higher in transgenic tobacco seedlings. When compared the samples after treatment, we can find that transgenic tobacco seedlings have a higher concentration of proline and soluble carbohydrates than the wild type, which demonstrated that overexpression of *JcERF2* in transgenic tobacco enhanced their tolerance to drought stress.

Discussion

J. curcas has great capability for environmental adaption, but the regulation mechanism of AP2/ERF genes in *J. curcas* is skimpy. Thus, the investigation of AP2/ERF genes in *J. curcas* is vital for understanding plant adaption to environmental stress. The expression of AP2/ERF transcription factors can be induced by a wide range of biotic and abiotic stresses, such as drought [14, 54], salt [54], hypoxia [15, 20, 31], pathogens [54], cold [41], submergence [14], freezing [53], potassium deficiency stress [28], and abscisic acid [46]. In our study, we observed that the transcript levels of *JcERF2* could be enhanced by salt, drought, ABA, and ethylene. Previous reports indicated that most ERF transcription factors act as transcriptional activators, but some function in transcriptional inhibition [52]. Thus, we predicted that a cross-talk signal pathway exists in *J. curcas* and *JcERF2* plays multiple roles in acquisition of plant stress tolerance. Investigation of the regulation and functional interactions, together with identification of new factors, is needed to understand the time-dependent action of *JcERF2* in a wide response network to environment stresses.

Plant hormone signaling pathways are reported to participate in a variety of developmental processes and stress response of environmental stimuli [57]. The stress treatment of ABA and ethylene induced increased expression of *JcERF2* at early stress period and then decreased, implicating that *JcERF2* may be involved in ABA-dependent stress responses. The ABA signaling pathways are reported to comprise signal transducers and transcription factors [58], but the information of their exacted interaction mechanism is limited. Further studies should be done to identify the core sequence and element protein binding with and to explain the complex regulation mechanisms of gene expression in response to changes in external and internal environments by *JcERF2* transcription factor. Besides, interactions between *JcERF2* and other proteins are also important to clarify the regulatory steps on gene expression.

External stress stimulates the expression of *JcERF2* and upregulates the expression of downstream genes involved in stress response, which improved the content of free proline and soluble sugar in plant [16, 22, 40]. The higher contents of proline in leaves of *JcERF2*-overexpressing lines under 30 % PEG conditions, suggesting the overexpression of *JcERF2* gene, enhance the proline accumulation and further increase the drought tolerance of transgenic tobacco by controlling the osmotic pressure of cells. We found that the amounts of free proline and soluble sugar are expressed through different pathway in transgenic tobacco. Before treatment, proline in transgenic tobacco showed a higher content more than wild type, which suggest that *JcERF2* may directly modulate the genes involved in the expression of proline, while *JcERF2* may regulate the expression of soluble sugar with the help of other

factors. From the qRT-PCR analysis, P5CS transcripts were higher in overexpression lines than wild type that confirmed that *JcERF2* overexpression lines increased the content of proline may due to the upregulation of *NtP5CS*. In our study, we also found that overexpression of *JcERF2* significantly upregulated the expression of a series of downstream genes (*NtOsmotin*, *NtPR1a*, *NtSAR8.2a*) in transgenic tobacco. Since the *JcERF2* gene could not be found in wild-type tobacco, we can draw the conclusion that these genes were modulated by JcERF*2*, which may contribute to the fact that these genes contain GCC boxes. The transgenic line exhibited a higher level of *NtSPSA* gene than wild type, but the soluble sugar showed the same level in both lines before treatment, which demonstrated that *JcERF2* affect sugar metabolism through different ways which include the expression of *NtSPSA* gene. The data presented in this report suggest that *JcERF2* is a positive transcription factor function in cross-talk between different stress pathways in plants. The transgenic tobacco showed a higher tolerance to drought, and salt stress indicated that *JcERF2* is an attractive engineering target in breeding plants for high antioxidant capacity to create multiresistant crops. However, the detail events involved in activating the defense response of *JcERF2* still need to be further researched to completely understand its function, since transgenic crops being the most controversial topics in recent years. In order to improve our understanding of *JcERF2*, RNAi plants need to be constructed; molecular markers and genomic transcriptome analysis also exhibit great advantage in deeply understanding the mechanism involved in *JcERFs*.

In conclusion, the novel ERF transcription factor *JcERF2* located in the nucleus and induced by exposure to salt, drought, ABA, and ethylene. Overexpression of *JcERF2* in transgenic tobacco showed increased tolerance to salt and drought stress. *JcERF2* upregulated the expression levels of *JcERF2*, *NtOsmotin*, *NtPR1a*, *NtSAR8.2a*, *NtP5CS*, and *NtSPSA* in transgenic tobacco, which indicated its multiple role in plant defense signal transduction networks.

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