



Research article

GhEIN3, a cotton (*Gossypium hirsutum*) homologue of AtEIN3, is involved in regulation of plant salinity tolerance

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ABSTRACT

Ethylene insensitive 3 (EIN3), a key transcription factor in ethylene signal transduction, play important roles in plant stress signaling pathways. In this study, we isolated and characterized an *EIN3*-like gene from cotton (*Gossypium hirsutum*), designated as *GhEIN3*. *GhEIN3* is highly expressed in vegetative tissues, and its expression is induced by 1-aminocyclopropane-1-carboxylic acid (ACC) and NaCl. Ectopic expression of *GhEIN3* in *Arabidopsis* elevated plants' response to ethylene, which exhibit smaller leaves, more root hairs, shorter roots and hypocotyls. The germination rate, survival rate and root length of *GhEIN3* transgenic plants were significantly improved compared to wild type under salt stress. *GhEIN3* transgenic plants accumulated less H₂O₂ and malondialdehyde (MDA), while higher superoxide dismutase (SOD) and peroxidase (POD) activities were detected under salt stress. In addition, expression of several genes related to reactive oxygen species (ROS) pathway and ABA signaling pathway was increased in the *GhEIN3* transgenic plants under salt stress. In contrast, virus-induced gene silencing (VIGS) of *GhEIN3* in cotton enhanced the sensitivity of transgenic plants to salt stress, accumulating higher H₂O₂ and MDA and lower SOD and POD activities compared to control plants. Collectively, our results revealed that *GhEIN3* might be involved in the regulation of plant response to salt stress by regulating ABA and ROS pathway during plant growth and development.

1. Introduction

Soil salinity is one of the most common environmental stresses affecting plant growth and crop productivity (Zhang et al., 2016a,b). The detrimental effects of salt on plants are a consequence of both a water deficit resulting in osmotic stress and the effects of excess sodium ions imposed on critical biochemical processes (Munns and Tester, 2008). The sessile nature of plants has favored the evolution of mechanisms to cope with salt stresses. Among these mechanisms, a number of stress-related genes directly protect the plants against salt stress, or either induce or repress the downstream target genes to regulate plant development and defense responses (Deinlein et al., 2014). Phytohormones and transcription factors (such as bZIP, WRKY, AP2/ERF, MYB, bHLH, and NAC families) regulate the expression levels of various stress-related genes, leading to plant tolerance to salt stress (Deinlein et al., 2014). In recent years, studies in *Arabidopsis* and rice have shown that phytohormone ethylene and its downstream transcription factors, including EIN3, AP2/ERF, play an important role in salt stress (Kazan,

2015; Peng et al., 2014; Yang et al., 2015; Zhang et al., 2011,2016a,b).

Ethylene is a simple gaseous phytohormone that affects a variety of basic plant processes throughout the whole plant growth and development from seed germination to organ senescence (Abeles et al., 1992; Liu et al., 2015; Van de Poel et al., 2015). In addition, ethylene plays a key role in plant responses to biotic and abiotic stresses (Kazan, 2015; Zhang et al., 2016a,b). Ethylene functions via five membrane-associated receptors to activate the downstream signaling pathway mediated by ETHYLENE INSENSITIVE3 (EIN3), and EIN3-like 1 (EIL1) (Chao et al., 1997; Solano et al., 1998; Merchante et al., 2013). The transcription factors EIN3 and EIL1, which act as the primary output of ethylene responses, activate or repress the expression of ethylene-response target genes by binding to their promoters and thereby modulate ethylene-related responses in plants (Boutrot et al., 2010; Chang et al., 2013; Feng et al., 2017; Kazan, 2015; Peng et al., 2014; Qiu et al., 2015; Solano et al., 1998; Zhang et al., 2011). Additionally, EIN3/EIL1 homologs in different plants had been isolated and characterized in recent years. Overexpression of *LeEILs* restored ripening in the ethylene

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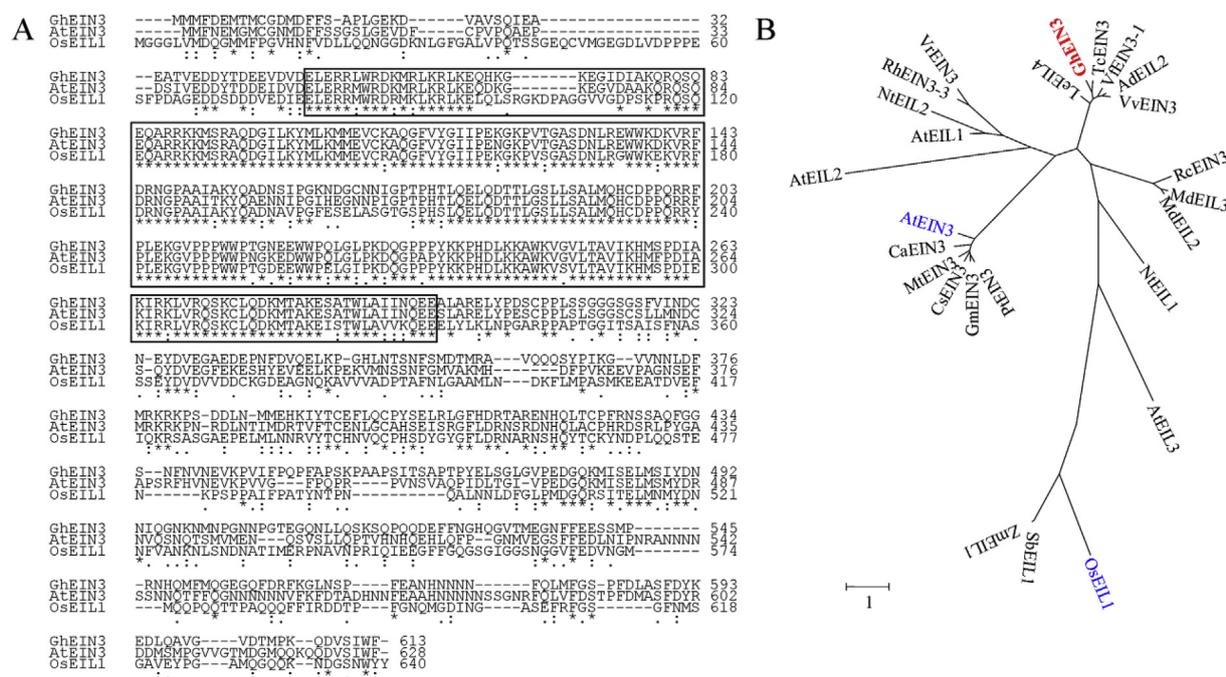


Fig. 1. Sequence alignment and phylogenetic analysis of EIN3 homologs. (A) Amino acid sequence alignments of EIN3 (*Arabidopsis thaliana*, AF004216) and OsEIL1 (*Oryza sativa Japonica*, AAZ78349) and GhEIN3 (*Gossypium hirsutum*, Gh_A05G0871). Identities and similarities between amino acid sequences are marked by star and dot, the EIN3 domain was showed by box. Numbers indicate the number of the amino acids from the first amino acid. (B) Phylogenetic relationship of GhEIN3 and other plant EIN3 homologs. The accession numbers of these known proteins in GenBank are: TcEIN3 (XP_007016682), RcEIN3 (XP_002530192), GmEIN3 (XP_003543159), LeEIL4 (NP_001233931), MdeIL2 (ADE41154), MdeIL3 (AGI41325), MtEIN3 (ACJ85584), NtEIL1 (AAP03997), NtEIL2 (AAP03998), OsEIL1 (AAZ78349.1), ADeIL2 (ACJ70675), CaEIN3 (XP_004501972), PteIN3 (XP_006384758), RHeIN3-3 (AGK07288), SbeIL (XP_002467935), CeEIN3 (AFK80347), VfeIN3-1 (ACD87815), VreIN (AF467783_1), VveIN3 (XP_002275284), ZmEIL1 (NP_001152035), AtEIN3 (AT3G20770), AtEIL1 (AT2G27050), AtEIL2 (AT5G21120), AtEIL3 (AT1G73730).

insensitive unripening tomato mutant (Li et al., 2012). Banana EIN3-like genes were differentially regulated by ethylene in mature green fruit (Mbéguié-A-Mbéguié et al., 2008). The rice *OsEIL1* and *OsEIL2* regulate ethylene response, wounding signaling and negatively affect salt tolerance (Hiraga et al., 2009; Yang et al., 2015). And *Oncidium EIL1* (*OgEIL1*) and *OgEIL2* mRNA levels in fully opened flowers increased as time progressed after cutting or exogenous application of ethylene (Chen et al., 2011). *Campanula EIL2* is a key gene in the ethylene signaling pathway, which is specific for the large flowered species *C. formanekiana* and *C. medium*. The naturally occurring 7 bp frameshift discovered in *Cmeil2*, correlates with ethylene insensitivity in flowers (Jensen et al., 2016).

Cotton, the largest source of fiber for textiles in the world, is considered to be a mild salt-tolerant crop, but salinity stress still has a great negative impact on its growth, yield and fiber quality, especially at germination and the young seedling stage (Ashraf, 2002; Zhou et al., 2014). Therefore, it is important to enhance cotton salt resistance through genetic manipulation. Some stress-responsive genes/proteins had been identified via transcriptome/proteomic profiling and successfully transferred to other plants or up/down-regulated in the cotton (Gong et al., 2017; Guo et al., 2015; Li et al., 2015; Yao et al., 2011). Several transcription factors play important roles in response to salt stress in cotton (Guo et al., 2009; He et al., 2016; Li et al., 2010; Ullah et al., 2018; Yan et al., 2014; Zhou et al., 2015). In this study, we characterized a cotton *EIN3*-like gene, *GhEIN3*, plays significant roles in salt stress tolerance. Ectopic expression of *GhEIN3* in *Arabidopsis* enhanced the transgenic plants' tolerance to salt stress. And GhEIN3 protein, as a transcriptional activator, may function in plant response to salt stress through regulating the ROS and ABA pathway.

2. Materials and methods

2.1. Plant materials and growth conditions

Cotton (*Gossypium hirsutum* cv. *Coker312*) seeds were surface-sterilized with 70% ethanol for 1 min and 10% H₂O₂ for 2 h, followed by washing with sterile water for three to five times. The sterilized seeds were germinated on half-strength Murashige and Skoog (MS) medium under a 16 h light/8 h dark cycle at 28 °C for 5 days. Roots, hypocotyls and cotyledons were cut from these sterile seedlings to extract total RNAs. Different cotton tissues including leaves, stems, petals, anthers, ovules and fibers were obtained from cotton plants for isolating total RNA. For ACC treatment, 5-day-old cotton seedlings were placed in MS liquid medium with 10 μM ACC for 0, 1, 2.5, 5, 10 h. The treated roots were harvested for RNA isolation. For *Arabidopsis thaliana* (*Col-0*) ethylene sensitivity assay, plant growth in air and ACC was carried out as described previously (Potuschak et al., 2003).

2.2. Gene cloning, sequence analysis and vector construction

The coding sequences of *GhEIN3* (Gh_A05G0871) were amplified by PCR and cloned into pBluescript II SK (pSK) vector for sequencing. The *GhEIN3* and its deduced proteins were analyzed using DNASTAR software (DNASTAR Inc., Madison, WI, USA), and protein sequence homology analysis was performed with Clustal omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). Phylogenetic analysis was employed to investigate the evolutionary relationships among the plant EIN3 homologs by MEGA 7.0 software with the bootstrap analysis conducted with 1000 iterations. Then, the coding sequence of *GhEIN3* in pSK vector was digested with different combination of restriction enzymes, and sub-cloned into *pCAMBIA2300/pCAMBIA2300-eGFP* to create *GhEIN3* overexpression vector and *GhEIN3:eGFP* vector.

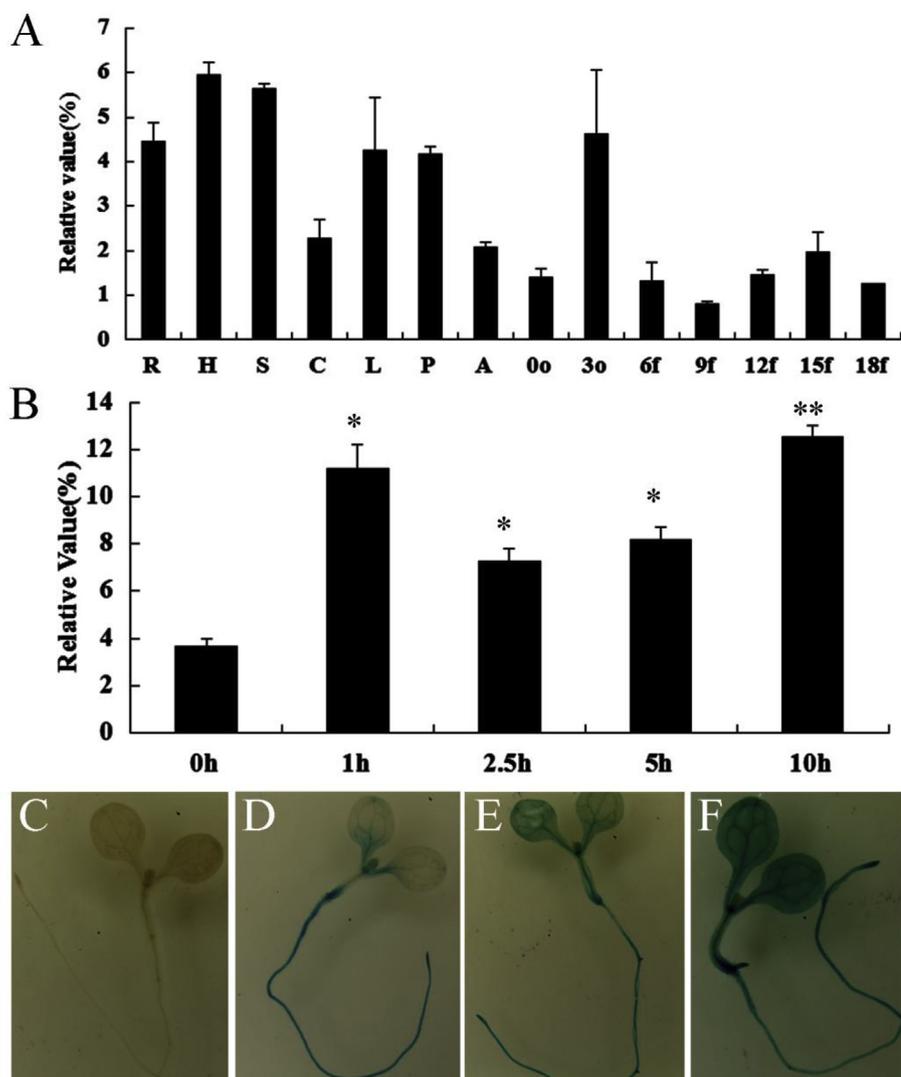


Fig. 2. Expression profile of *GhEIN3* in cotton tissues/organs and induced by ACC and NaCl treatment. (A) Expression patterns of *GhEIN3* in different cotton tissues/organs. R: Root; H: Hypocotyl; S: Stem; C: Cotyledon; L: Leaf; P: Petal; A: Anther; 0o: 0 days post anthesis (DPA) ovule; 3o: 3 DPA ovule; 6f: 6 DPA fiber; 9f: 9 DPA fiber; 12f: 12 DPA fiber; 15f: 15 DPA fiber; 18f: 18 DPA fiber. (B) Expression analysis of *GhEIN3* after ACC treatment. Relative values of *GhEIN3* expression in cotton tissues are shown as a percentage of *GhUBI1* expression activity. Independent *t*-tests demonstrated that there was significant difference (* $P < 0.05$) in *GhEIN3* expression level between the treated seedlings and controls. (C–F) GUS Staining analysis of *GhEIN3Pro-GUS* Transgenic *Arabidopsis* treated with 150 mM NaCl. C: Wild type; (D, E, F) *GhEIN3Pro-GUS* transgenic *Arabidopsis* treated with 150 mM NaCl for 0 h, 10 h and 20 h, respectively.

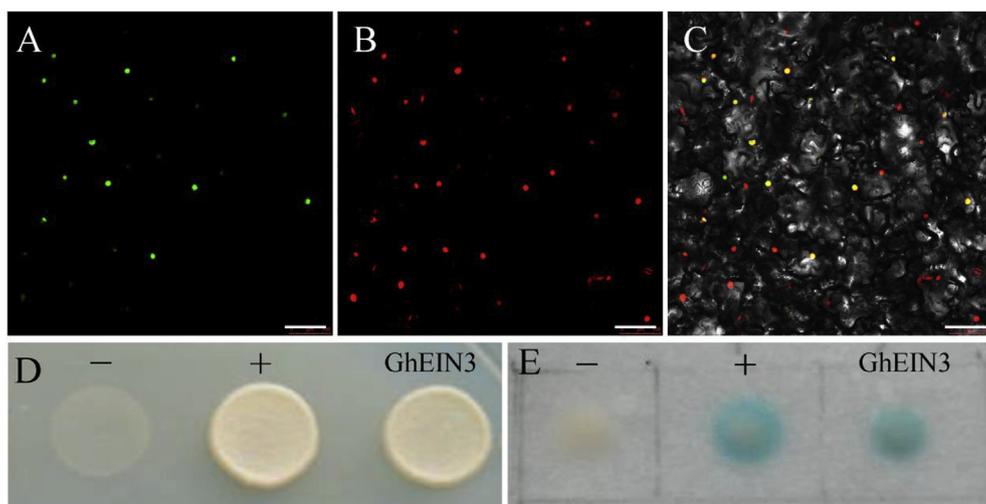


Fig. 3. Subcellular localization and transactivation activity assay of *GhEIN3*. (A) GFP fluorescence. (B) DAPI (a nuclear-specific dye) fluorescence. (C) The image GFP and image DAPI merged with its bright-field photograph in the same cell. Scale bar = 100 μ m. (D) Yeast transformants grew on SD/-Trp/-His/-Ade medium. (E) X-gal filter assay of the β -galactosidase activity. -: Yeast transformants of pGBKT7 was used as the negative control. +: Yeast transformants of pGBKT7-53/pGADT7-RecT was used as the positive control. *GhEIN3*: Yeast transformants of pGBKT7-*GhEIN3*.

2.3. Subcellular location of *GhEIN3* protein

The *GhEIN3:eGFP* vector was transferred into *Agrobacterium tumefaciens* GV3101 and transiently expressed in tobacco (*Nicotiana benthamiana*) leaves as described previously (Sparkes et al., 2006). Subsequently, GFP fluorescence and DAPI fluorescent staining tobacco leaf

epidermal cells were detected under a SP5 Meta confocal laser microscope (Leica, Wetzlar, Germany).

2.4. Transactivation activity assay

For transcriptional activation assay, the coding sequence of *GhEIN3*

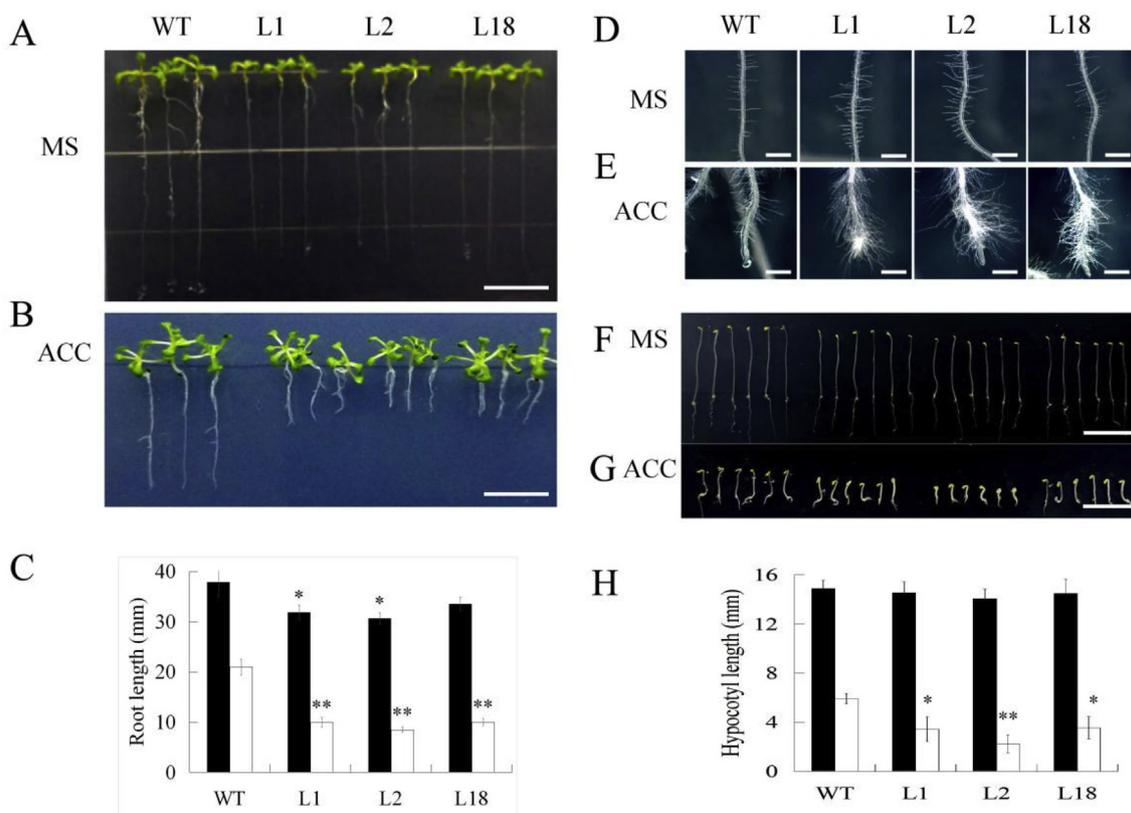


Fig. 4. Phenotypic analysis of overexpression *GhEIN3* transgenic *Arabidopsis*. (A–B) 12-day-old transgenic plants (L1, L2 and L18) showed shorter primary root length than the wild type on MS medium supplemented with 10 mM ACC, while they showed similar primary root length on MS medium without ACC. (C) Measurement and statistical analysis of primary root length of (A, B). (D, E) 5-day-old transgenic plants (L1, L2 and L18) had longer and higher density of root hair, compared to wild type plants on MS with/without 10 μ M ACC. (F, G) 5-day-old transgenic plants (L1, L2 and L18) showed shorter hypocotyls than that of wild type in dark with 10 mM ACC, while they showed similar hypocotyl length on MS medium without ACC. (H) Measurement and statistical analysis of hypocotyls length of (F, G). Error bars represent \pm SD of at least three biological replicates (* $P < 0.05$, ** $P < 0.01$). (A, B, F, G) Scale bar = 10 mm, (D, E) Scale bar = 2 mm.

was cloned into pGBKT7 vector (Clontech, Palo Alto, CA, USA) and transferred into yeast strains AH109 and Y187 using the high-efficiency lithium acetate transformation procedure. AH109 transformants were grown on double dropout medium (SD/-Ade/-Trp) and Y187 transformants were further confirmed with color change on a β -galactosidase filter paper using the flash-freezing filter assay (Zhang et al., 2010). The yeast harboring empty pGBKT7 and pGBKT7-53 vector was used as the negative and positive control, respectively.

2.5. Quantitative RT-PCR (qRT-PCR) analysis

The cotton total RNA was extracted and digested with RNase-free DNase I using the RNApure Plant Kit (TIANGEN, Beijing, China). Then, 2–3 μ g of total RNA was used for reverse transcription by M-MLV Reverse Transcriptase (Promega, USA). Expression of *GhEIN3* in cotton different tissues was analyzed by qRT-PCR using the fluorescent intercalating dye SYBR Green in a detection system (Opticon2; MJ Research) as described previously (Li et al., 2005). A cotton ubiquitin gene (*GhUBI1*, GenBank accession number: EU604080) was used as a quantitative control in qRT-PCR reactions. Each qRT-PCR analysis was performed for three replicates. The primers were showed in Table S1.

Arabidopsis total RNA was isolated from the seedlings by Trizol reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instruction. qRT-PCR analysis was performed to check the expression of several salt stress-associated genes, using *AtActin2* as a normalization control. Each qRT-PCR analysis was performed for three replicates. The primers were showed in Table S1.

2.6. *Arabidopsis* transformation and seed germination assay

35S:*GhEIN3* vector was transformed into *Arabidopsis thaliana* (Col-0) by the floral dip method (Zhang et al., 2006). Three independent homozygous lines of the T3 and T4 generations were chosen for phenotypic analysis. The wild type *Arabidopsis* and *GhEIN3* transgenic seeds were sterilized and grown on MS medium with different concentrations of NaCl. The seeds were placed in dark at 4 $^{\circ}$ C for two days to break seed dormancy, and then transferred in a plant growth incubator (22 $^{\circ}$ C, 16 h light/8 h dark cycle) to let seeds germinate. A seed was considered as germinated as long as its radicle penetrated the seed coat. The experiments were carried out with three biological replicates and each replicate represents 40 seeds for each line.

2.7. Root growth assay

Seeds of wild type and *GhEIN3* overexpressing transgenic *Arabidopsis* were germinated on MS media for 4 days (22 $^{\circ}$ C, 16 h light/8 h dark cycle) and then seedlings which had no significant difference in primary root length were transferred to MS media with or without NaCl. The primary root length was measured at fifth day after transferred. The experiments were carried out with three biological replicates and each replicate represents 20 seeds for each line.

2.8. VIGS of *GhEIN3* gene in cotton

To investigate the putative function of *GhEIN3* in cotton, VIGS experiments were performed according to Gao et al. (2013) with minor modifications. Target gene fragments of *GhEIN3* and *GhCLA1*

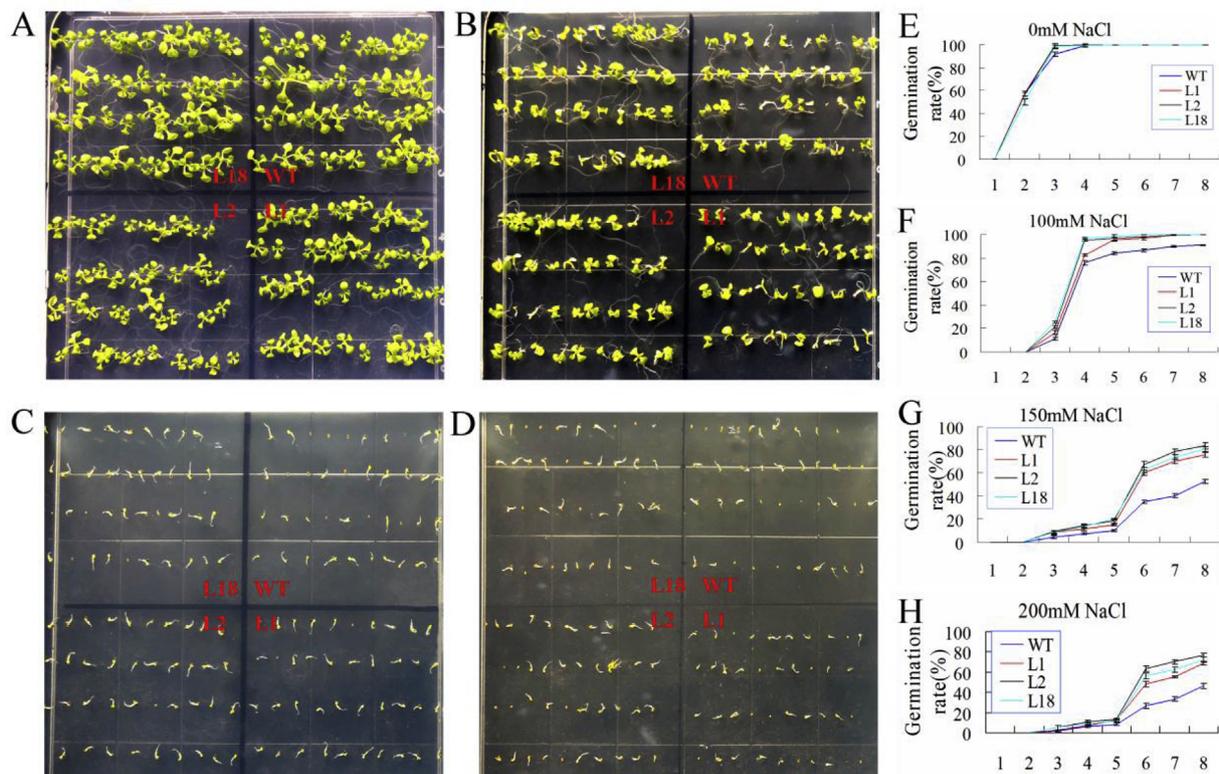


Fig. 5. Overexpression of *GhEIN3* in *Arabidopsis* enhance germination rate under salt stress. (A–D) Wild type and transgenic seedlings grow in MS medium, 100 mM NaCl, 150 mM NaCl and 200 mM NaCl, respectively. (E–H) Measurement and statistical analysis of germination rate of *GhEIN3* transgenic and wild type seedlings correspond to A–D. (F–H) Independent *t*-tests demonstrated that there was significant difference ($P < 0.05$) in germination rate in 5–8 days between the *GhEIN3* transgenic lines and controls.

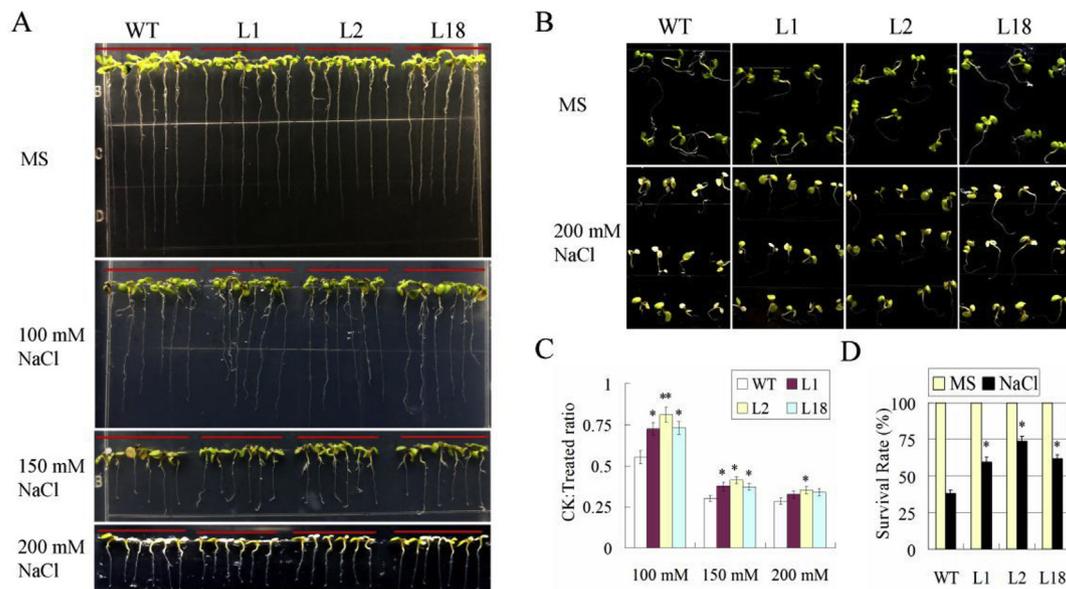


Fig. 6. Overexpression of *GhEIN3* in *Arabidopsis* enhance root length and survival rate under salt stress. (A, C) Transgenic plants showed better roots growth than wild type in the presence of 100, and 150 mM NaCl. (B, D) Transgenic plants showed higher survival rate than the wild type in the presence of 200 mM NaCl. Error bars represent \pm SD of at least three biological replicates (* $P < 0.05$, ** $P < 0.01$).

(chloroplastos alterados 1, positive control) were amplified from the *G. hirsutum* leaf cDNA to generate *TRV:GhEIN3* and *TRV:GhCLA1* vectors (the PCR primers were shown in Table S1). Then, the *TRV:GhEIN3* and *TRV:GhCLA1* vectors were transformed into *Agrobacterium tumefaciens* (GV3101) by electroporation. Agro-infiltration was performed using a needleless syringe on 8-day-old seedlings for *G. hirsutum* coker 312. The

inoculated seedlings were then transferred to a climate chamber and grown at 25 °C in the dark for 2 days and then exposed to light (16 h/8 h light/dark) for 2 week. qRT-PCR was performed to further confirm that *GhEIN3* had been silenced in VIGS experiments. Finally, these *GhEIN3*-silenced plants and control plants were treated by 400 mM NaCl solution regularly after every 4 days until the phenotypes appeared.

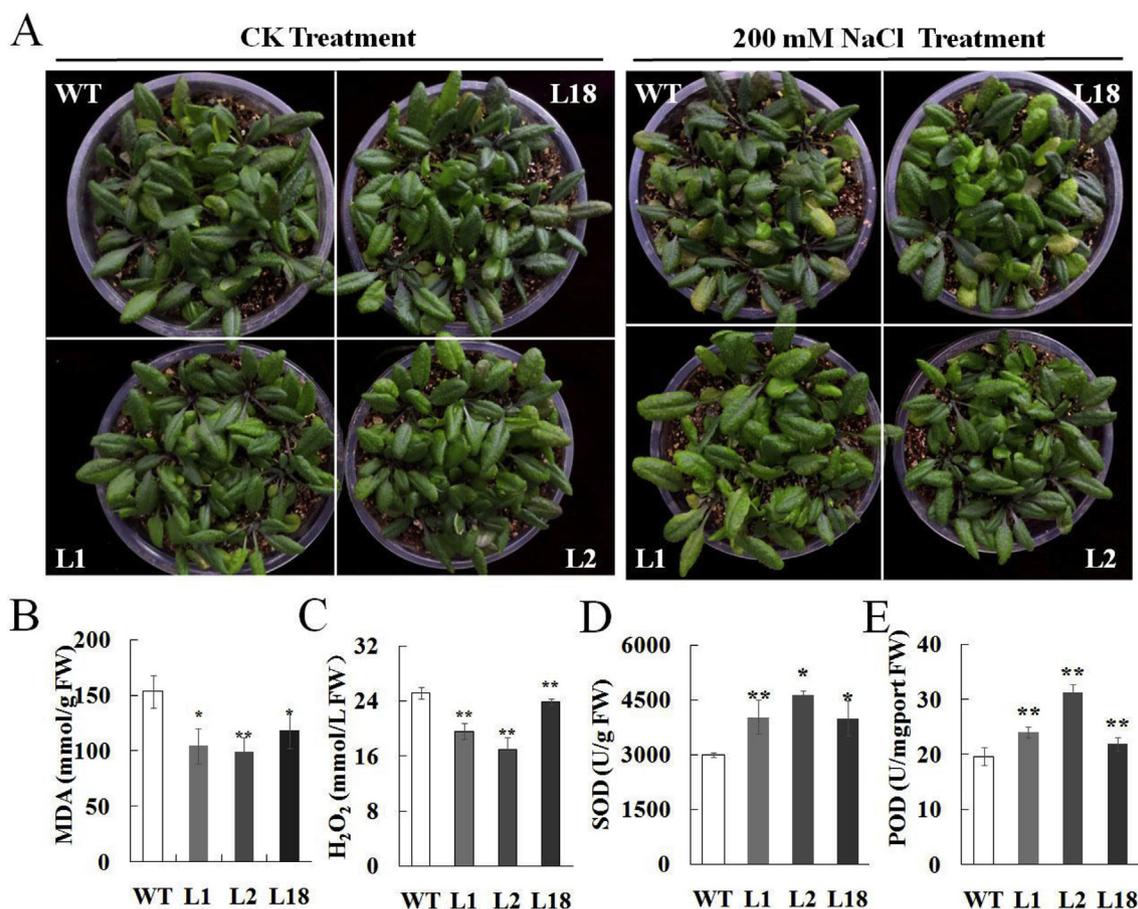


Fig. 7. Overexpression of *GhEIN3* in *Arabidopsis* enhance Salt-tolerance in soil. (A) *GhEIN3* transgenic lines showed better growth with lower H₂O₂ and MDA content (B, C), more SOD and POD activities (D, E) in the presence of 200 mM NaCl in soil condition. Error bars represent \pm SD of at least three biological replicates (* $P < 0.05$, ** $P < 0.01$).

2.9. Determination of MDA, H₂O₂ content and SOD, POD activity

To check malondialdehyde (MDA), hydrogen peroxide (H₂O₂) superoxide dismutase (SOD) and peroxidase (POD) activity in *Arabidopsis* leaves, one group of 4-week-old *GhEIN3* transgenic plants and wild type *Arabidopsis* plants were subjected to salt stress by giving 200 mM NaCl solution for 5 days whereas another group plants were kept untreated as controls. For detecting MDA, H₂O₂ content and SOD, POD activity in cotton leaves, one group of 5-week-old *TRV:GhEIN3* and *TRV:00* cotton plants were subjected to salt stress by giving 400 mM NaCl solution whereas another group plants were kept untreated as controls. To detect MDA, H₂O₂ content, and SOD and POD activity, 0.1 g leaves were collected from the *Arabidopsis* plants and cotton plants. Leaves were ground in liquid nitrogen was used for the determination of these contents. For quantification of MDA and H₂O₂ contents, leaves of *Arabidopsis* or cotton were prepared and followed the procedure as described in plant MDA assay kit (Colorimetric method) and hydrogen peroxide assay kit (Nanjing Jiancheng Bioengineering Institute, China), respectively. The measurement of POD and SOD enzyme activity were followed the procedure as described in Peroxidase assay kit and Superoxide Dismutase (SOD) assay kit (Nanjing Jiancheng Bioengineering Institute, China), respectively.

3. Results

3.1. Identification and sequence analysis of *GhEIN3*

To study whether ethylene signaling regulators are involved in

cotton salt stress, we identified sixteen EIN3-like (EIL) genes in cotton (*Gossypium hirsutum*) genome (Fig. S1). Among them, *GhEIN3* (Gh_A05G0871) displays relatively higher expression activity in vegetative tissues (<http://structuralbiology.cau.edu.cn/gossypium/>), and was selected as a candidate to investigate its function in salt stress of cotton. Sequence analysis indicated that *GhEIN3* has an open reading frame of 1842 bp in length with no introns, and encodes a protein of 613 amino acids. Multi-sequence alignment analysis results showed the DNA-binding domain of *GhEIN3* in the N-terminus is highly conserved with *AtEIN3* and *OsEIL1* (Fig. 1A). Phylogenetic analysis and DNASTAR software analysis revealed that *GhEIN3* shared a higher identity with *AtEIN3* (68.7%), *AtEIL1* (69.7%) than *Arabidopsis thaliana* *EIL2* (43.3%), and *AtEIL3* (42.1%) (Fig. 1B). Additionally, *GhEIN3* also shared 50.5%–89.9% identity with other EIN3 homologs, including *Theobroma cacao* *TcEIN3* (XP_007016682, 89.9%), *Oryza sativa* *OsEIL1* (AAZ78349.1, 51.5%), *Zea mays* *ZmEIL1* (NP_001152035, 50.5%), *Ricinus communis* *RcEIN3* (XP_002530192, 80.9%), *Glycine max* *GmEIN3* (XP_003543159, 76.3%), *Lycopersicon esculentum* *LeEIL4* (NP_001233931, 69.8%), *Malus domestica* *MdEIL2* (ADE41154, 64.9%) and *MdEIL3* (AGI41325, 63.9%), *Medicago truncatula* *MtEIN3* (ACJ85584, 74%), *Nicotiana tabacum* *NtEIL1* (AAP03997, 73.5%) and *NtEIL2* (AAP03998, 73.2%), *Actinidia deliciosa* *AdeEIL2* (ACJ70675, 72.7%), *Cicer arietinum* *CaEIN3* (XP_004501972, 73.2%), *Populus trichocarpa* *PtEIN3* (XP_006384758, 78.4%), *Rosa hybrid cultivar* *RhEIN3-3* (AGK07288, 78.1%), *Sorghum bicolor* *SbEIL* (XP_002467935, 51.3%), *Cucumis sativus* *CsEIN3* (AFK80347, 77.3%), *Vicia faba* *VfEIN3-1* (ACD87815, 73.8%), *Vigna radiata* *VrEIN3* (AF467783_1, 73.1%), and *Vitis vinifera* *VvEIN3* (XP_002275284, 71.8%) (Fig. 1B). Phylogenetic

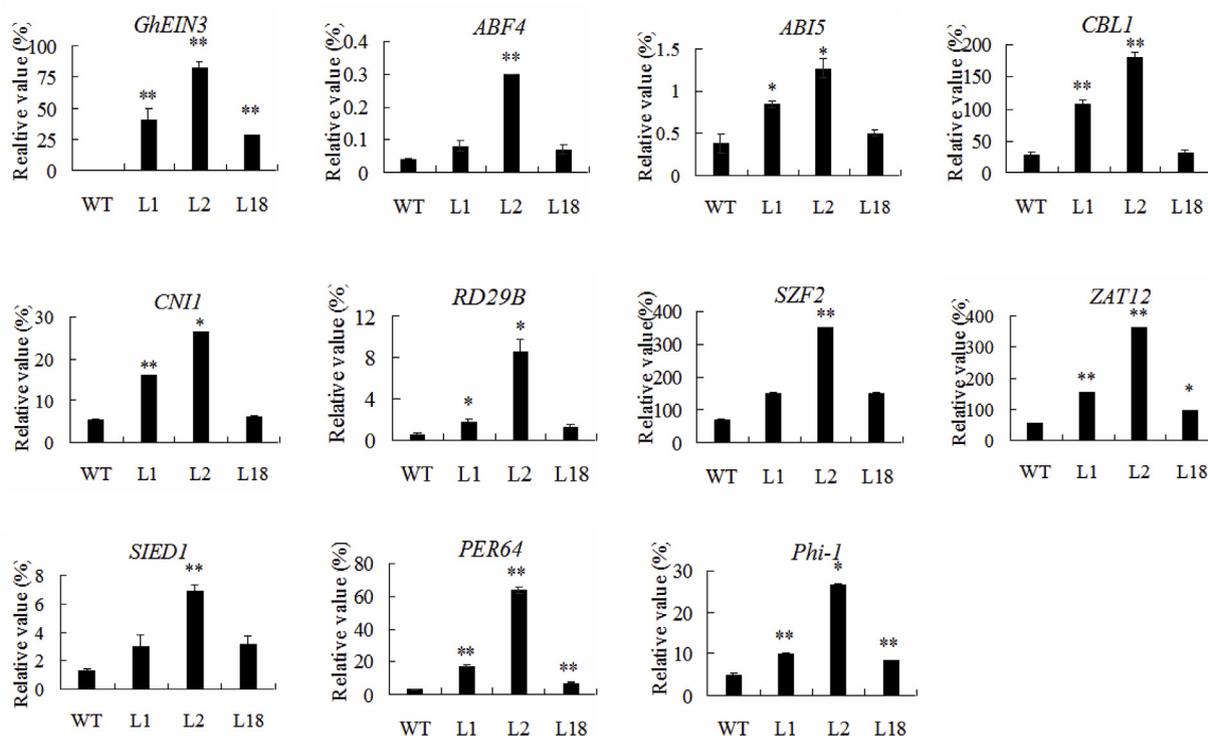


Fig. 8. Relative expression of reference genes under salt stress in transgenic *Arabidopsis*. The expression level of ABA signaling pathway and ROS-responsive genes in *GhEIN3* transgenic plants under salt stress. Transcript levels of *GhEIN3*, *ABF4*, *ABI5*, *CBL1*, *CN11*, *RD29B*, *SZF2*, *ZAT12*, *SIED1*, *PER64*, and *Phi-1* genes were determined by quantitative RT-PCR, using *AtActin2* as a quantification control. Error bars represent \pm SD of three biological replicates (* $P < 0.05$, ** $P < 0.01$).

relationship analysis revealed that *GhEIN3* belongs to EIN3/EIL1 subfamily and has the closest relationship with *Theobroma cacao* EIN3.

3.2. Expression of *GhEIN3* induced by ACC and NaCl

To investigate the spatial expression of *GhEIN3* in cotton, qRT-PCR was performed. The results showed that *GhEIN3* is expressed in all tissues of cotton. The transcripts of *GhEIN3* accumulated higher in roots, hypocotyls, stems, leaves, petals and 3dpa ovules than in other tissues (Fig. 2A).

Since *GhEIN3* is a homologue of *AtEIN3*, which participates in ethylene signaling, we presume its expression may be induced by ACC, an ethylene biosynthesis precursor. Five-day-old cotton seedlings were treated with ACC and then total RNAs were isolated from the roots of these treated cotton seedlings for quantitative RT-PCR analysis (Fig. 2B). The results showed that the expression of *GhEIN3* was significantly up-regulated in cotton seedlings by ACC.

To investigate the relationship between *GhEIN3* and salt stress, we analyzed *GhEIN3* promoter's activity using transgenic *Arabidopsis* carrying a fusion of *GhEIN3* promoter and *GUS* gene. *GUS* activity was detectable mainly in roots of 5 days after germination (DAG) seedlings (Fig. 2D). *GUS* activity in roots increased continuously after the seedlings being transferred to MS with 150 mM NaCl medium for 10 h or 20 h (Fig. 2E–F). The results indicated that the expression of *GhEIN3* can be induced by NaCl.

3.3. *GhEIN3* is a transcriptional activator

To determine whether *GhEIN3* is a transcriptional activator, we transferred the *pGBKT7-GhEIN3* construct into yeast strain *AH109* and *Y187*, using *pGBKT7* vector as a negative control. The transformed yeast cells with *GhEIN3* grew well on the selective SD/-Trp/-Ade medium, indicating that the reporter gene *ADE2* was activated (Fig. 3A). Additionally, the transformed yeast cells harboring *GhEIN3*

turned blue in the presence of X-Gal, indicating that the reporter gene *LacZ* was activated. By contrast, yeast cells harboring empty *pGBKT7* vector did not grow on the selective medium, and did not turn blue (Fig. 3B).

Additionally, we generated the transformed tobacco leaf cells transiently expressing *GhEIN3:GFP*. GFP signals in cells were detected only in the cell nucleus (Fig. 3C–E). These results suggest that *GhEIN3* protein functions as an active transcriptional activator.

3.4. Ectopic expression of *GhEIN3* in *Arabidopsis* enhances plants response to ethylene

To further investigate the functions of *GhEIN3*, we cloned and transformed it into *Arabidopsis* via a floral dip method. After the RNA extraction, qRT-PCR confirmed that *GhEIN3* had been successfully transformed into *Arabidopsis*, and line L1, L2, L18 were selected for further analysis. Growing in the normal culture, the transgenic *GhEIN3* seedlings showed slightly smaller leaves, shorter roots and hypocotyls, compared with wild type plants (Fig. S2, Fig. 4A, C, F, H). After the treatment of 10 mM ACC, the root length and hypocotyl length of wild type plants decreased obviously. Meanwhile, the *GhEIN3* overexpressing transgenic plants were more sensitive to ACC treatment which showed much shorter roots and hypocotyls than that of the wild type under ACC treatment (Fig. 4B, C, G, H). In addition, *GhEIN3* overexpressing transgenic plants showed longer root hair and higher root hair density in normal condition compared to wild type (Fig. 4D). Interestingly, the root hair density and length of *GhEIN3* transgenic plants increased more significantly than wild type under ACC treatment (Fig. 4E). These results indicated that *GhEIN3*, acts as a positive regulator, participates in ethylene signaling.

3.5. Overexpressing *GhEIN3* plants enhanced salt tolerance

Arabidopsis EIN3 positively regulates salt stress tolerance, while rice

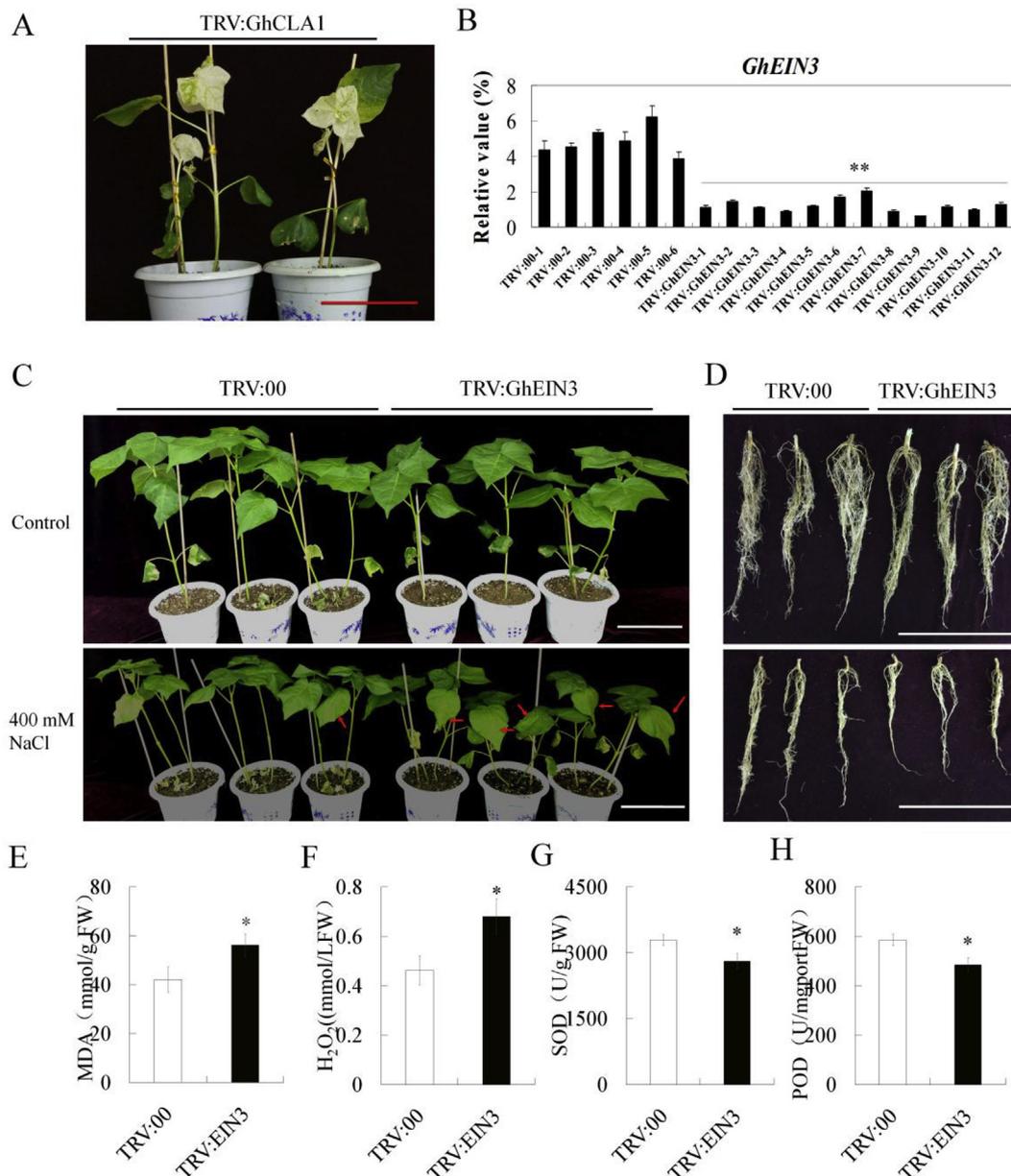


Fig. 9. *GhEIN3* VIGS plants enhanced sensitivity to salt by ROS pathway. (A) Bleaching phenotype of a TRV:*GhCLA1* plants maintained for five weeks. (B) qRT-PCR analysis confirmed that *GhEIN3* has been silenced in TRV:*GhEIN3* cotton seedlings. Relative values of *GhEIN3* expression in cotton leaves are shown as a percentage of *GhUB1* expression activity. Independent *t*-tests demonstrated that there was significant difference (***P* < 0.01) in *GhEIN3* expression level between the TRV:*GhEIN3* plants and TRV:00 control plants. (C) TRV:*GhEIN3* plants showed higher sensitivity to 400 mM salt stress compared to TRV:00 control plants. The red arrow showed the relative wilt leaves. (D) TRV:*GhEIN3* plants exhibited shorter roots compared to TRV:00 under 400 mM salt stress. (E, F) TRV:*GhEIN3* cotton leaves showed higher H₂O₂ and MDA content. (G, H) TRV:*GhEIN3* cotton leaves showed lower SOD, POD activities compared to TRV:00 leaves in the presence of 400 mM NaCl in soil condition. Error bars represent \pm SD of three biological replicates (**P* < 0.05). Scale bar = 10 cm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

EIL1/EIL2 negatively affects salt stress tolerance (Peng et al., 2014; Yang et al., 2015). To gain insight into the effects of *GhEIN3* on salt stress tolerance, the transgenic seed germination capacity on MS agar medium containing different concentrations of NaCl was analyzed. As shown in Fig. 5A, E, less difference in seed germination was observed between the *GhEIN3* overexpressing transgenic lines and wild type without NaCl treatment. When the concentration of NaCl was increased to 100 mM, the germination rates of *GhEIN3* overexpressing seeds was slightly higher (about 5–10% higher) than the wild type seeds at 8 d after sowing (Fig. 5B, F). *GhEIN3* overexpressing transgenic lines showed dramatically higher germination rates than wild type on the MS medium containing 150 and 200 mM NaCl (Fig. 5C, D, G, H). As the

NaCl concentration was further increased to 150 mM, germination rates of three *GhEIN3* transgenic lines were 75% (L1), 83% (L2) and 80% (L18), respectively, while wild type was only 54% at the eighth day after sowing (Fig. 5G). And on MS medium with 200 mM NaCl, seed germination rates of three *GhEIN3* transgenic lines were 72% (L1), 80% (L2) and 79% (L18), respectively, while wild type was only 49% at the eighth day after sowing (Fig. 5H). Statistical analysis showed significant differences in seed germination between the transgenic plants and wild type.

Meanwhile, we also examined the primary root length of overexpressing *GhEIN3* plants and wild type under salt stress (Fig. 6). On MS medium, we found the primary root lengths of the *GhEIN3* transgenic

plants are shorter than wild type slightly. Under 100 and 150 mM NaCl treatments, however, primary root length of *GhEIN3* transgenic plants was longer than that of wild type, especially on MS with 100 mM NaCl (Fig. 6A, C). Additionally, a 200 mM concentration of NaCl inhibited the growth of both transgenic plants and wild type, but the transgenic plants showed higher green leaf rate (Fig. 6A). Then, we conducted survival rate assay to further evaluate the role of *GhEIN3* under salt stress. Plants were grown on MS medium for 5 d and then transferred onto MS medium supplemented with 200 mM NaCl for 3 d. The over-expressing *GhEIN3* plants showed a higher survival rate compared to wild type (Fig. 6B, D). In addition, we further evaluate the role of *GhEIN3* under salt stress in soil and checked the antioxidant enzyme activity of transgenic plants and wild type (Fig. 7). The results showed *GhEIN3* transgenic plants enhanced salt-tolerance compared to wild type (Fig. 7A). Salt stress can increase the accumulation of reactive oxygen species (ROS), which results in cell damage. To evaluate the role of *GhEIN3* in the oxidative stress pathway, MDA and H₂O₂ contents were checked in *GhEIN3* transgenic plants and wild type. The results showed MDA and H₂O₂ accumulated less in transgenic plants than wild type under NaCl stress (Fig. 7B and C). For evaluating the ROS-scavenging capability in *GhEIN3* transgenic plants and wild type, SOD and POD activities were also checked. The SOD and POD activities were higher in transgenic plants compared to wild type under NaCl stress (Fig. 7D and E). These results *GhEIN3* can enhance plant tolerance to salt by decreasing the accumulation of reactive oxygen species (ROS).

ABA is a plant hormone that mediates the adaptation of plants to abiotic stresses, including salt stress. To determine the signaling pathways in which overexpressing plants obtained tolerance against salt stress, relative expression of the ABA signaling pathway (*AtABF4*, *AtABI5*, *CNI1*, *AtCBL1* and *AtRD29B*), and reactive oxygen species (ROS) pathway (*SIED1*, *SZF2*, *ZAT12*, *PER64* and *Phi-1*) were checked in transgenic *Arabidopsis* and wild type under salt stress (Kang et al., 2002; Msanne et al., 2011; Tezuka et al., 2013; Peng et al., 2014; Zhang et al., 2016a,b). Our results showed that the relative expression of these genes was significantly higher in transgenic lines compared to wild type under salt stress (Fig. 8). These results indicated that overexpression of *GhEIN3* in *Arabidopsis* increased salt stress tolerance during seedling development by regulating the ABA and ROS pathway.

3.6. Enhanced salt sensitivity in *GhEIN3*-VIGS plants

To further investigate the functions of *GhEIN3* in cotton, a VIGS assay of *GhEIN3* was applied to cotton Coker 312, with *GhCLA1* as a reporter gene to evaluate the efficiency of VIGS. After inoculation with *Agrobacterium tumefaciens* (GV3101) contained the *TRV:GhEIN3* or *TRV:00* or *TRV:GhCLA1*, respectively, we obtained twelve *GhEIN3*-silenced plants, six *TRV:00* control plants, and six positive (*TRV:GhCLA1*) plants. After 14 days of inoculation, the albino phenotype of *TRV:GhCLA1* plants was found (Fig. 9A). And the expression of *GhEIN3* in *TRV:00* and *TRV:GhEIN3* plants was checked to confirm whether the corresponding gene expression had been reduced or not. The results showed that *GhEIN3* had been effectively silenced in the *TRV:GhEIN3* plants compared to *TRV:00* plants (Fig. 9B). Then, *TRV:00* and *TRV:GhEIN3* plants were subjected to salt stress. After 21 days of salt treatment, the shorter shoots and roots were found in *TRV:GhEIN3* plants compared to control plants (Fig. 9C and D, red arrow). *TRV:GhEIN3* plants also appeared relatively wilt leaves compared to control plants (Fig. 9C, red arrow). These results revealed that silence of *GhEIN3* can enhance plant sensitivity to salt.

To evaluate the role of *GhEIN3* in the oxidative stress pathway, we measured MDA and H₂O₂ contents under salt stress in *TRV:GhEIN3* and *TRV:00* plants. Under salt stress, a greater accumulation of MDA and H₂O₂ was observed in *TRV:GhEIN3* plants compared to *TRV:00* plants (Fig. 9E and F). This result revealed that *TRV:GhEIN3* plants were more severely damaged by ROS compared to the control plants under salt stress. To check the ROS-scavenging capability in *TRV:GhEIN3* and

TRV:00 plants, SOD and POD activities were also checked. A lower SOD and POD activities were observed in *TRV:GhEIN3* plants compared to control plants (Fig. 9G and H). The results suggested that *TRV:GhEIN3* plants have a weaker ability to scavenge peroxides and therefore they are more sensitive to salt stress.

4. Discussion

EIN3/EIN3-like 1 (EIL1) homologs are positive regulators of ethylene signal transduction pathway and had been widely characterized in different species of plants. However, there is no report about *EIN3* or *EIN3*-like genes (*EILs*) in cotton so far. In this study, an *EIN3* homologous gene (*GhEIN3*) from cotton was isolated and characterized. Sequence alignment showed that *GhEIN3* is homologous with the reported *EILs* in different plant species (Fig. 1), indicating a high conservation of the *EIL* family among different plant species. *GhEIN3* was expressed in different cotton tissues, with a relatively high expression level in vegetative tissues. The transcript of *GhEIN3* was also induced by ACC treatment. It has been demonstrated that overexpression of *AtEIN3* and *OsEIL1* genes activate ethylene response (Chao et al., 1997; Mao et al., 2006). In our experiments, overexpression of *GhEIN3* in *Arabidopsis* also activated the ethylene signaling, resulting in longer root hair and higher root hair density which suggested that the basic function of the *EIN3/EILs* is conserved (Potuschak et al., 2003). Sequence alignment analysis showed that high sequence identity was existed among at the EIN3 domain of the *GhEIN3*, *AtEIN3* and *OsEIL1* (Fig. 1).

Salinity is undoubtedly a major abiotic stress factor that restricts plant productivity by causing ionic, oxidative, and osmotic stress (Kazan, 2015). When plants encounter salt stress, one important cause of high salinity-imposed damage is ROS generated by salt stress. On one hand, ROS plays a dual role in plants as modulators of cellular signaling pathways. On the other hand, it acts as oxidative agents or toxic products elicited by cellular stresses (Apel et al., 2004; Shabala et al., 2015). ROS is tightly regulated by the equilibrium between production and scavenging (Perez and Brown, 2014). In *Arabidopsis*, the expression of several genes (*ZAT12*, *SZF2*, *SIED1*, *PER64* and *phi-1*) related to ROS pathway was notably elevated in *AtEIN3ox* plants under salt condition compared with that in wild type or *ein3 eil1* (Peng et al., 2014). Besides, Peng et al. (2014) found lower levels of H₂O₂ (a kind of ROS) accumulation in wild type plants but higher levels in *ein3 eil1* upon salt treatment (Peng et al., 2014). Similarly, Zhang et al. (2016a,b) reported that tobacco ethylene response factor 1 (TERF1), which regulated by EIN3-like proteins, functions in ROS scavenging (Zhang et al., 2016a,b). In our studies, we found that ectopic expression of *GhEIN3* in *Arabidopsis* up-regulated the expressions of *ZAT12*, *SZF2*, *SIED1*, *PER64* and *phi-1*, and accumulated lower levels of H₂O₂ and MDA, exhibited higher activities of POD and SOD under salt treatment. The result was in accordance with *AtEIN3*'s role in salt stress. Furthermore, in our study, *GhEIN3*-VIGS plants accumulated higher levels of H₂O₂ and MDA, but exhibited lower activities of POD and SOD under salt treatment (Fig. 9). The MDA level under stress conditions is an indicator of ROS destructive effects, while SOD and POD are antioxidant enzymes which can scavenge the toxic ROS and lead to enhanced tolerance against different abiotic stresses (Gill and Tuteja, 2010). Lower SOD and POD activities were detected in *GhEIN3*-VIGS plants, which reveal that *GhEIN3*-VIGS plants were less protected from oxidative and osmotic damages. Higher accumulation of MDA and H₂O₂ content in *GhEIN3*-VIGS plants suggested that these *GhEIN3* knockdown plants have a less efficient system of scavenging ROS. Our results are consistent with those recent advances, which showed that the ethylene signaling modulates salinity responses largely via regulation of ROS-scavenging mechanisms (Jiang et al., 2013; Peng et al., 2014; Zhang et al., 2016a,b). So *GhEIN3*, likely to the *Arabidopsis* *EIN3*, plays positive roles in plant salt tolerance by ROS pathway. In rice, however, *OsEIL1* and *OsEIL2* negatively regulate salt tolerance (Yang et al., 2015). And this negative regulation by *OsEIL1* and *OsEIL2* in salt tolerance is likely

attributable to the direct regulation of HIGH-AFFINITY K⁺ TRANSPORTER2; 1 expression and Na⁺ uptake in roots (Yang et al., 2015). Sequence alignment analysis showed that the similarity between GhEIN3 and AtEIN3 was higher than that of OsEIL1/2, especially at the C-terminus of these EIN3 homologs (Fig. 1). So the C-terminus of the EIN3 may play an important function in plant salt stress response.

ABA is a plant hormone that mediates the adaptation of plants to unfavorable environmental conditions including salinity and drought (Shabala et al., 2015). ABA play a crucial role in root development under salt stress (Chen et al., 2016; Ullah et al., 2018; Zhu et al., 2017), ABA also maintains seed dormancy, prevents germination and inhibits seedling growth (Finkelstein and Lynch, 2000; Tezuka et al., 2013). And salt stress is able to induce ABA biosynthesis and trigger ABA-dependent signaling pathways (Zhu, 2002). Several studies showed ABA and ethylene can crosstalk under salt stress (Silva et al., 2018; Yu et al., 2016; Kissoudis et al., 2017). As so far, it had not been reported whether AtEIN3/EIL1 are involved in ABA-mediated salt stress response. EIN3/EIL1 were positive regulator of ethylene signaling pathway, which also positively regulates salt stress tolerance by activating *ERF1*, *AP2/ERF ETHYLENE AND SALT INDUCIBLE 1 (ESE1)* and *CNI1* (Peng et al., 2014). The downstream genes of ERF1 and ESE1 include *RD20*, *RD29A*, *COR15A*, and *RD29B* (Cheng et al., 2013; Solano et al., 1998), which are also downstream genes of ABA signaling pathway (Aubert et al., 2011 Nakashima et al., 2006; Wilhelm and Thomashow, 1993). In recent years, several studies showed that ERFs can positively regulate salinity tolerance via regulating ABA signaling (Yao et al., 2016; Li et al., 2018, 2019). This prompted us to come up with the idea that EIN3 regulates plant tolerance to salt stress partly via regulation of ABA signaling pathway. In our studies, *GhEIN3* overexpressing *Arabidopsis* plants had a better germination rate and longer roots under salt stress, while the *GhEIN3-VIGS* cotton plants show shorter roots under salt condition (Fig. 6; Fig. 8). Furthermore, the expression of several genes (*AtABF4*, *AtAB15*, *CNI1*, *AtCBL1* and *AtRD29B*) related to ABA pathway was notably elevated in *GhEIN3* overexpressing plants under salt condition compared with wild type. Therefore, it is likely that EIN3 regulates seed germination rate and root length under salt stress through ABA signaling pathway.

In summary, GhEIN3 was involved in ROS and ABA-mediated process of salt tolerance by up-regulating transcripts of downstream stress-responsive genes of ROS pathway and ABA pathway. Hereby, we hypothesized that *GhEIN3* participates in the stress signaling pathway, and therefore, according to our understanding, the overexpression of *GhEIN3* will increase salt tolerance in cotton as well as in other crops.

Author's contributions

GQH, XQW and LHH conceived and designed the experiment, and performed most of the experiments. QQH, WZ, MT, WYW and YNZ performed some of the experiments and assisted in data analysis. XBL, DDL and GQH analyzed the data and wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2019.08.022>.

Competing financial interests

The author(s) declare no competing financial interests.

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