Mulberry EIL3 confers salt and drought tolerances and modulates ethylene biosynthetic gene expression

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ABSTRACT
Ethylene regulates plant abiotic stress responses and tolerances, and ethylene-insensitive (EIN3)/EIN3-like (EIL) proteins are the key components of ethylene signal transduction. Although the functions of EIN3/EIL proteins in response to abiotic stresses have been investigated in model plants, little is known in non-model plants, including mulberry (Morus L.), which is an economically important perennial woody plant. We functionally characterized a gene encoding an EIN3-like protein from mulberry, designated as MnEIL3. A quantitative real-time PCR analysis demonstrated that the expression of MnEIL3 could be induced in roots and shoot by salt and drought stresses. Arabidopsis overexpressing MnEIL3 exhibited an enhanced tolerance to salt and drought stresses. MnEIL3 overexpression in Arabidopsis significantly upregulated the transcript abundances of ethylene biosynthetic genes. Furthermore, MnEIL3 enhanced the activities of the MnACO1 and MnACS1 promoters, which respond to salt and drought stresses. Thus, MnEIL3 may play important roles in tolerance to abiotic stresses and the expression of ethylene biosynthetic genes.

Key words: Mulberry, MnEIL3, Arabidopsis, Abiotic stresses, Salt stress, Drought.

INTRODUCTION
Ethylene is gaseous hormone that regulates many physiological processes, including seed germination, seedling growth, leaf expansion, flower opening, senescence, and fruit development. Ethylene is synthesized from methionine by a catalysis mediated by S-adenosyl-L-methionine synthetase, 1-aminocyclopropane-1-carboxylic acid synthase (ACS), and 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) (Kende, 1993; Yang & Hoffman, 1984). ACS and ACO are the rate-limiting enzymes that regulate ethylene biosynthesis, and several regulators influence ethylene production by changing the activities and expression levels of ACS and ACO.

The ethylene perception and signal transduction pathway has been well studied in model plants, like Arabidopsis thaliana, tomato (Solanum lycopersicum), and tobacco (Nicotiana tabacum). In the presence of ethylene, the endoplasmic reticulum-localized receptors (ethylene
receptors, ETRs) perceive ethylene, resulting in the inactivation of constitutive triple response 1, which is the negative regulatory factor of ethylene responses (Clark et al., 1998; Hua et al., 1998; Rodríguez et al., 1999). Then, ethylene insensitive 2 (EIN2), an essential positive regulator of ethylene signaling, is dephosphorylated, and its carboxyl terminus is cleaved and enters into the nucleus, where it binds to ethylene insensitive 3/EIN3-like proteins (EIN3/EILs) (Chao et al., 1997; Qiao et al., 2012). Eventually, the activated EIN3/EILs regulate the transcription of ethylene-responsive factors (ERFs) and other downstream genes (Alonso & Stepanova, 2004; Wang et al., 2002).

EIN3/EILs are the key elements that initiate the ethylene-mediated downstream transcriptional cascade (An et al., 2018). The mutation of EIN3/EILs genes, AtEIN3 (AT3G20770) and AtEIL1 (AT2G27050), result in ethylene-insensitive performance, and plants overexpressing AtEIN3 and AtEIL1 show enhanced ethylene production and triple responses in Arabidopsis. The ein3-1 eil1-1 double mutant completely abolishes the ethylene response in etiolated Arabidopsis seedlings (Chao et al., 1997; Alonso et al., 2003). The stabilities of EIN3/EILs are regulated by EIN3-binding F-box proteins (EBF1 and EBF2) in the EBF1- and EBF2-mediated ubiquitin-proteasome degradation pathway, and mutations of EBF1 and EBF2 accumulate EIN3/EIL proteins and display constitutive ethylene responses (Potuschak et al., 2003). Ethylene quickly stabilizes EIN3/EIL1 by promoting EBF1 and EBF2 proteasomal degradation, which contributes to the ethylene responses (An et al., 2010). In addition, the M KK9-M PK3/MPK6 cascades promote EIN3-mediated transcription in ethylene signaling by regulating the phosphorylation and protein stability of EIN3 (Yoo et al., 2008).

The EIN3/EILs family are plant-specific transcription factors (TFs) and bind to primary ethylene response elements (PEREs) and EIL conserved binding sequences (ECBSs) in the promoters of downstream genes involved in the response to ethylene (Yin et al., 2010). Thus, EIN3/EILs regulate many physiological processes, including apical hook formation, hormone responses, fruit development, abiotic stress responses, seedling photomorphogenesis, and light perception, by activating the expression of a wide range of downstream genes (An et al., 2012;
He et al., 2011; Zhu et al., 2011; Shan et al., 2012; Peng et al., 2014; Shi et al., 2012; Shi et al., 2018). Recently, studies have focused on the functions of EIN3/EILs in abiotic stress tolerances. Peng et al. (2014) demonstrated that EIN3/EIL1 are essential for the enhanced ethylene-induced salt tolerance in Arabidopsis, and salt stress stabilizes EIN3/EIL1 proteins by promoting EBF1/EBF2 proteasomal degradation in an EIN2 independent manner. In addition, a large number of EIN3/EIL1-regulated genes that participate in salt stress responses have been identified using whole-genome transcriptome analyses, including many genes encoding reactive oxygen species scavengers. An AP2 domain-containing gene, ESE1, is an ethylene-modulated gene downstream of EIN3/EIL1 in the salt response (Zhang et al., 2011). Mutations of EIN3 increase the sensitivity in response to water stress stimulated by polyethylene glycol (PEG) 6000 (Cui et al., 2015). Genetic and biochemical analyses revealed that EIN3 proteins act as negative factors against freezing stress by repressing the expression of C-repeat binding factors and type-A Arabidopsis response regulator (ARR) 5, ARR7, and ARR15 (Shi et al., 2012). The functions of the EIN3/EIL1 proteins in response to heavy metal stresses have also been studied. Kong et al. (2018) found that cadmium (Cd) inhibits EIN3 protein degradation in Arabidopsis, and the ein3-1 eil1-1 double mutant plants display an increased tolerance to Cd. EIN3 enhances root growth inhibition under Cd stress by regulating the expression of the xyloglucan endotransglucosylase/hydrolase 33 and response to low sulfur 1 genes, which are involved in cell wall modification and sulfur metabolic processes, respectively (Kong et al., 2018).

Mulberry (Morus L.) is an economically important perennial woody plant belonging to Moraceae of Rosales, which have multiple uses in silkworm rearing, ecology, pharmaceuticals, and traditional Chinese medicines (He et al., 2013). Mulberry adapts well to drought, salinity, water logging, and other abiotic stress conditions, but little is known regarding the molecular mechanisms of the tolerance. In our previous studies, the elements involved in mulberry ethylene biosynthesis and signal transduction were identified and its functions in fruit development were clarified (Liu et al., 2014; Liu et al., 2015). However, the functions of mulberry ethylene biosynthesis and signal pathway related genes in other aspects of the lifecycle remain unclear,
especially in abiotic stress responses and tolerances. In this study, we investigated the physiological functions of a mulberry gene encoding EIN3-like proteins, \textit{MnEIL3}, in salt and drought tolerances by analyzing its expression patterns and its heterologous overexpression in \textit{Arabidopsis}. \textit{MnEIL3}'s expression was significantly upregulated by salt and drought stresses, and its overexpression in \textit{Arabidopsis} led to enhanced salt and drought stress tolerances and the upregulated expression of ethylene biosynthetic genes. Furthermore, \textit{MnEIL3} significantly enhanced the activities of \textit{MnACO1} and \textit{MnACS1} promoters. Thus, a working model for \textit{MnEIL3} in plant tolerance to abiotic stresses was suggested.

**MATERIALS AND METHODS**

**Plant materials and growth conditions**

\textit{A. thaliana} ecotype Columbia-0 and the \textit{ein3-1 eil1-1} mutant were used as plant materials and were grown at 24°C/22°C under a 16-h light/8-h dark photoperiod.

A mulberry (\textit{M. notabilis} Schneid) tree, which was used for genome sequencing, is an isolated wild mulberry species with a chromosome number of 14. The seedlings of \textit{M. notabilis} were used in this study and grown in a PQX-type plant incubator with artificial intelligence capability (Ningbo Southeast Instrument Corporation, China) under a 16-h light/8-h dark photoperiod at 26°C/22°C (day/night). For stress treatments, the one-month-old seedlings were subjected to salt [0.6 % (m/v) NaCl] and drought [20 % (m/v) PEG6000]. The roots and shoot of the treated seedlings were sampled at 0, 1, 3, 6, 12, and 24 h post-treatment. The 14-d-old seedlings were treated independently with 200 mM NaCl and 200 mM mannitol, and the treated seedlings were sampled at 0, 1, 3, 6, and 12 h post-treatment. The harvested materials were frozen immediately in liquid nitrogen for total RNA extraction.

**RNA extraction and quantitative real-time PCR (qRT-PCR)**

Total RNA extraction, first-strand of cDNA synthesis, and qRT-PCR analysis were performed as described in our previous study (\textit{Wei et al., 2014}). The \textit{ACTIN3} and \textit{β-actin2} genes were used as internal controls for mulberry and \textit{Arabidopsis}, respectively, and the relative expression was
defined as $2^{-[C_{t}(\text{target gene})-C_{t}(\text{control gene})]}$. All qRT-PCRs were performed with three independent biological replicates. The primers used are specified in Table S2.

**Plasmid construction**

The full-length coding sequence of *MnEIL1* (GenBank accession number: XM_010107825) and *MnEIL3* (XM_010093690) were cloned into the *Nco*I and *Bgl*II restriction sites of the pCAMBIA1302 expression vector under the control of the CaMV35S promoter, and *MnEIL2* (XM_010107826) was cloned into the *Bgl*II and *Spe*I restriction sites of the pCAMBIA1302 expression vector. Finally, the *CaMV35S::MnEIL1*, *CaMV35S::MnEIL2*, and *CaMV35S::MnEIL3* recombinant plasmids were generated.

The 5′ upstream regions of the *MnACS1* and *MnACS3* genes were independently inserted into the *Eco*RI and *Nco*I restriction sites of the pCAMBIA1301 expression vector, producing the *MnACS1pro::GUS* and *MnACS3pro::GUS* recombinant plasmids, respectively. The primers used are specified in Table S2.

**Plant transformation**

The recombinant plant expression vectors were transformed into *Agrobacterium tumefaciens* strain GV3101. *MnEIL3*, *MnACS1pro::GUS* and *MnACS3pro::GUS* were eventually independently transformed into *A. thaliana* (Columbia-0) using the floral dip method (Clough & Bent, 1998). The transgenic *Arabidopsis* lines were evaluated by GUS staining, genomic PCR, inverse PCR, and qRT-PCR analyses. The homozygous lines of the T3 generation were used for further research.

**Stress treatments of transgenic *Arabidopsis***

Wild type, *ein3-1eil1-1*, and *MnEIL3* transgenic seeds were germinated on 1/2 Murashige and Skoog (MS) agar medium. The 7-d-old seedlings were transferred into pots containing the soil supplemented with normal nutrients were grown at 24°C/22°C under a 16-h light/8-h dark photoperiod. The 21- and 14-d-old seedlings were treated with salt [1.2 % (m/v) NaCl] and drought (watering treatments withheld), respectively, and the proline, hydrogen peroxide (H$_2$O$_2$),
and malondialdehyde (MDA) contents, were measured using their respective test kits (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions. Each treatment was replicated three times.

The seeds of the transgenic plants that contained MnACS1pro::GUS and MnACS3pro::GUS were germinated on 1/2 MS agar medium. The 10-d-old seedlings were exposed to salt (200 mM NaCl) and drought (200 mM mannitol) treatments. The samples were subjected to GUS staining after 0, 1, 3, 6, and 12 h of exposure.

**Transient expression assays and GUS activity detection**

CaMV35S::MnEIL1, CaMV35S::MnEIL2, and CaMV35S::MnEIL3 recombinant plasmids were used as effector plasmids, and MnACO1pro::GUS, MnACO2pro::GUS, MnACS1pro::GUS, and MnACS3pro::GUS recombinant plasmids were used as reporter plasmids. Of these vectors, the MnACO1pro::GUS and MnACO2pro::GUS recombinant plasmids have been reported in a previous study (Yu et al., 2017). The reporter and effector plasmids were transformed into A. tumefaciens strain GV3101. The bacteria were mixed and co-injected into the strawberry fruit as described in a previous study (Spolaore et al., 2001). The injected tissues were sampled and used for the GUS staining analysis. Meanwhile, the GUS activity of the injected tissues were detected by 4-nitrophenyl-β-D-glucopyranoside methods (Jefferson, 1987).

**Statistical analyses**

The statistical analyses methods were as described in a previous study (Liu et al., 2017a). All data were conducted using SPSS statistical software 17.0 (SPSS Inc., Chicago, IL, USA) and Excel 2013 (Microsoft, Redmond, CA, USA). The results are presented as mean values ± SEs. The significant differences between samples were analyzed using a one-way ANOVA in SPSS Statistics 17.0. The analyses of significant differences ($P < 0.05$) were measured by Student’s $t$-test analysis.

**RESULTS**

**Expression patterns of MnEIL genes under salt and drought stresses**
The expression levels of *MnEIL* genes under NaCl and PEG treatments were assessed by qRT-PCR. Under salt stress, the transcript abundance of *MnEIL1* was significantly upregulated and downregulated in roots and shoots, respectively, 3 h after the NaCl treatment, but the expression levels after 24 h were not different than those at 0 h (Figs. 1A and 1B). The expression of *MnEIL2* in roots was significantly upregulated after 1 h of NaCl treatment. *MnEIL2*’s expression in shoots was downregulated at 1, 6, and 12 h, but its expression was upregulated at 24 h after the NaCl treatment (Figs. 1A and 1B). The transcript abundances of *MnEIL3* in roots and shoot were significantly upregulated 3 h after the NaCl treatment, but the expression of *MnEIL3* in shoots after 24 h showed no difference with that at 0 h (Figs. 1A and 1B). Under drought stress conditions, *MnEIL1*’s expression levels in roots and shoot were downregulated by the NaCl treatment, although *MnEIL1*’s expression in roots was upregulated at 3 h (Figs. 1C and 1D). The transcript abundance of *MnEIL2* was significantly upregulated and downregulated after 3 h of NaCl treatment in roots and shoot, respectively (Figs. 1C and 1D). The expression of *MnEIL3* in roots was significantly upregulated after a PEG treatment, although its expression showed no response to PEG at 12 h. *MnEIL3*’s expression in shoots was significantly upregulated 6 h after the PEG treatment and exhibited a strong expression peak at 24 h (Figs. 1C and 1D).

**The overexpression of *MnEIL3* in *Arabidopsis* enhances salt and drought tolerances**

The *MnEIL3* gene was selected for further investigation base on its responses to salt and drought stresses. The full-length sequence of the *MnEIL3* gene was inserted into the pCAMBIA1302 vector under the control of the *CaMV35S* promoter and transformed into wild type *Arabidopsis* plants. Transgenic lines were obtained using hygromycin resistance and confirmed by genomic PCR and qRT-PCR analyses (Figs. S1A and S1B). In addition, the insertion site of the transgene construct was determined using inverse PCR. The *CaMV35S::MnEIL3* recombinant plasmid was inserted into chromosome 2 of the *Arabidopsis* genome (Fig. S1C).

To evaluate the tolerance of *MnEIL3*-overexpressing (*MnEIL3ox*) plants against salt stress, the 21-d-old wild type, *MnEIL3ox*, and *ein3-1 eil1-1 Arabidopsis* seedlings were treated with 1.2% (m/v) NaCl. After treatment, *MnEIL3ox* plants showed relatively greater growth rates than wild
type and ein3-1 eil1-1 Arabidopsis. Additionally, the ein3-1 eil1-1 seedlings showed a decreased salt tolerance compared with wild type plants (Figs. 2A and 2B). To characterize the performance of MnEIL3ox plants under drought stress, the 14-d-old seedlings of wild type, MnEIL3ox, and ein3-1 eil1-1 Arabidopsis plants were treated with drought stress. The growth of wild type, MnEIL3ox, and ein3-1 eil1-1 plants showed no difference under drought stress conditions. However, MnEIL3ox showed a greater capability to survive than wild type and ein3-1 eil1-1 plants when the treated plants were re-watered (Figs. 2A and 2B).

To understand the mechanism behind the enhanced sensitivity to drought and salt stresses caused by MnEIL3’s overexpression, the accumulated levels of H$_2$O$_2$, MDA, and proline were analyzed. The MDA and H$_2$O$_2$ contents in MnEIL3ox and ein3-1 eil1-1 plants were lower and higher than in wild type Arabidopsis, respectively (Figs. 2C and 2D). The proline contents in MnEIL3ox and ein3-1 eil1-1 plants were higher and lower than in wild type Arabidopsis, respectively (Figs. 2C and 2D). Thus, MnEIL3 may negatively regulate drought and salt stress tolerances.

The enhanced expressions of ACS and ACO genes in MnEIL3ox plants

In this study, the expression levels of ACS- and ACO-encoding genes were detected in MnEIL3ox plants. The transcript abundances of AtACS4/6/8/10/12 genes in MnEIL3ox plants were higher than in wild type Arabidopsis, while the expression levels of AtACS1/7 genes showed were lower in MnEIL3ox plants. Moreover, there was no difference in the expression of AtACS3 between MnEIL3ox and wild type plants (Fig. 3). All of the detected AtACO genes, AtACO1/2/4, showed higher expression levels in MnEIL3ox plants than in wild type Arabidopsis (Fig. 3).

Mulberry EIL proteins modulate MnACO1, MnACO2, MnACS1, and MnACS3 promoter activities

To explore the correlations between the expression of MnEIL3 and ethylene biosynthetic genes, MnACO1, MnACO2, MnACS1, and MnACS3 were selected for promoter isolation. All these genes have been determined as the key genes involved in ethylene biosynthesis in mulberry (Liu
et al., 2014; Liu et al., 2015). The gene’ promoters were downloaded from the Morus genome database (http://morus.swu.edu.cn/morusdb/) and isolated from mulberry (M. notabilis). By searching for cis-acting regulatory elements, PERE- and ECBS-binding sites were identified in the promoters of MnACO1, MnACO2, MnACS1, and MnACS3 (Table S1). In vivo interactions between MnEIL3 and these promoters were estimated by transient analyses in strawberry fruit. MnEIL3 significantly enhanced the activities of MnACO1 and MnACS1 promoters, while no significant effects on the activities of MnACO2 and MnACS3 promoters were found (Fig. 4). Thus, EIL proteins may act as the transcriptional activators of ethylene biosynthetic genes. We also detected correlations between the other two MnEIL genes, MnEIL1/2, and ethylene biosynthetic genes. Thus, MnEIL1 acted as the activator of MnACO2 and MnACS3 promoters, while MnEIL2 regulated the activities of the MnACO1 and MnACS3 promoters (Fig. 4).

The activities of MnACSI and MnACS3 promoters were regulated by salt and drought stresses

To examine the responsiveness of the MnACSI and MnACS3 genes under salt and drought stresses, transgenic Arabidopsis were generated by introducing MnACSIpro::GUS- and MnACS3pro::GUS-fused genes, and then the 10-d-old seedlings were exposed to stresses (Fig. 5A). The MnACSI promoter in leaves responded to NaCl and mannitol treatments (Fig. 5A). The GUS reporter in the MnACS3pro::GUS transgenic Arabidopsis was mainly expressed in roots, while little GUS accumulation levels in stems and leaves were detected. Under salt stress, the GUS accumulation levels in the stems of MnACS3pro::GUS transgenic Arabidopsis were enhanced after 6 h of NaCl treatment, while the GUS levels in stems and leaves were enhanced after 3 h of mannitol treatment (Fig. 5A).

The expression levels of MnACSI and MnACS3 are regulated by salt and drought stresses

The 14-d-old seedlings were treated with NaCl and mannitol, and then used to detect the expression levels of MnACSI and MnACS3. MnACSI’s expression level was significantly upregulated after 1 h of NaCl treatment, and its expression was transiently upregulated after 1 h of mannitol treatment and then was downregulated (Figs. 5B and 5C). The transcript abundance
of MnACS3 was significantly upregulated after 3 h of NaCl treatment, and its expression was upregulated after 3 h of mannitol treatment, but it showed no response at any other time point (Figs. 5D and 5E).

**DISCUSSION**

EIN3/EILs proteins are positive factors in ethylene signal transduction. In model plants, EIN3/EILs are involved in many aspects of the life cycle, including seed germination, soil emergence, seedling development, leaf senescence, pigments biosynthesis, light perception, and abiotic stress responses (Kim et al., 2017; Zhong et al., 2014; An et al., 2018; Yu et al., 2013; Yu et al., 2016). Additionally, the regulatory functions of EIN3/EILs in response to abiotic stresses have also attracted considerable attention (Zhang et al., 2011; Cui et al., 2015; Shi et al., 2012; Kong et al., 2018; Peng et al., 2014). However, there are no reports on the functions of EIN3/EILs in the abiotic stress tolerance of woody plants, including mulberry. In the present study, the expression levels of mulberry MnEIL genes under salt and drought stresses were revealed, and they showed different patterns. Among these genes, the expression of MnEIL3 was significantly upregulated by salt and drought stresses in roots and shoots (Fig. 1), which is similar to the expression patterns of Arabidopsis AtEIN3 and AtEIL1 genes (Fig. S2). The full-length coding sequence of MnEIL3 was transformed into Arabidopsis for stress tolerance analysis. The expression levels of MnEIL3 were significantly upregulated in roots and shoots by NaCl treatments (Fig. 1), and the overexpression of this gene in Arabidopsis enhanced salt stress tolerance (Fig. 2). MnEIL3’s overexpression decreased the MDA and H$_2$O$_2$ contents and enhanced the proline content under salt stress. Thus, MnEIL3 positively regulated plant salt tolerance, which was similar to the results described by Peng et al (2014). In this study, ein3-1 eil1-1 plants showed decreased tolerances to drought stress compare with wild type plants (Fig. 2), and mutation of EIN3/EILs decreased the MDA and H$_2$O$_2$ contents and enhanced the proline content under drought stress. This was similar to the results of a previous study which reported that the ein3-1 mutant exhibited a decreased tolerance to drought stress stimulated by PEG6000 (Cui et al., 2015). In addition, MnEIL3ox plants showed a greater ability to survive drought
stress than wild type and ein3-1 eil1-1 plants. Thus, MnEIL3 may play a positive role in abiotic stress tolerances, and it indicates that the functions of EIL3/EILs in response to abiotic stresses are relatively conserved in plants.

The overexpression of kiwifruit (Actinidia deliciosa) EIN3-like transcription factors, AdEIL2 and AdEIL3, increased ethylene production by upregulating the expression of ACS and ACO genes in transgenic Arabidopsis (Yin et al., 2010). Based on the data reported by Liu et al. (2017b), several genes that were involved in ethylene biosynthesis and signal transduction showed lower expression levels in ein3-1 eil1-1 plants compared with wild type Arabidopsis. Here, the transcript abundances of AtACS and AtACO genes were mainly upregulated in MnEIL3ox plants. The analysis suggested the positive feedback regulation of EIN3/EILs in ethylene production.

When plants receive the ethylene signal, EIN3/EILs are activated, and then, they regulate the transcriptional expression of downstream responsive genes, including ERF, ACO, xyloglucan endo-transglycosylase, and cell wall-modifying genes (Solano et a, 1998; Huang et al., 2010; Yin et al., 2010; Ireland et al., 2014). The PERE and ECBS motifs in promoters have been identified as EIN3-interactive motifs. MnACO1, MnACO2, MnACS1, and MnACS3 promoters contain PERE and ECBS motifs, which suggests that ethylene biosynthetic genes can be regulated by EIN3/EILs. In the present study, we found that MnEIL3 and two other MnEILs (MnEIL1 and MnEIL2) modulate the activities of MnACO1 and MnACO2 promoters as assessed by transient analysis in strawberry (Fragaria × ananassa Duch.) fruit. This result was similar to those reported in kiwifruit and melon (Cucumis melo L. cv. Andes) (Yin et al., 2010; Huang et al., 2010). MnACS1 and MnACS3 promoter’ activities were regulated by MnEIL1/2/3 (Fig. 4). Thus, MnEIL proteins provide positive feedback regulation during ethylene production by directly regulating the transcription of ethylene biosynthetic genes. We also constructed MnACS1pro::GUS and MnACS3pro::GUS vectors and independently introduced them into Arabidopsis. The GUS activities in the stems and leaves of the transgenic Arabidopsis seedlings were enhanced by salt and drought stresses (Fig. 5A). Additionally, the expression levels of
MnACS1 and MnACS3 significantly responded to salt and drought stresses (Figs. 5B-5E). The promoter’ activities and gene expression levels of MnACO1 and MnACO2 were also enhanced by abiotic stresses (Yu et al., 2017).

On the basis of our results, we proposed a working model for the regulatory network of mulberry MnEIL3 in response to abiotic stresses (Fig. 6). When plants are exposed to abiotic stresses, the stress signals are perceived by plant cells, leading to the enhanced accumulation of ethylene. Then, the ethylene signal transduction pathway was induced and the nucleus-localized MnEIL3 proteins’ accumulation and stability were enhanced. MnEIL3’s expression was also significantly induced by stresses. MnEIL3 eventually positively regulates abiotic stress tolerances by activating downstream stress-responsive genes. In addition, MnEIL3 binds to the target regions in the promoters of ACO and ACS genes, and activates gene expression, which contributes to the accumulation of ethylene. MnEIL1 and MnEIL2 may function in other processes, such as fruit development and maturation, by modulating ethylene responses (Liu et al., 2015). However, more work is needed to investigate the roles of the ethylene–EIN3/EILs–ACO/ACS regulatory loop in abiotic stress tolerances.

CONCLUSIONS

In summary, our results explored the functions of a gene encoding an EIN3-like protein from mulberry, MnEIL3. The expression level of MnEIL3 significantly increased in response to salt and drought stresses in roots and shoot. Transgenic Arabidopsis overexpressing MnEIL3 exhibited an enhanced tolerance to salt and drought stresses. The overexpression of MnEIL3 significantly upregulated the expression levels of ethylene biosynthetic genes in Arabidopsis. Moreover, MnEIL3 could enhance the activities of MnACO1 and MnACS1 promoters, which suggested an ethylene–EIN3/EILs–ACO/ACS regulatory loop in abiotic stress tolerance. This research provides insights into the functions of MnEIL3 in abiotic stress tolerance and their influence on the expression levels of ethylene biosynthetic genes.

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**ADDITIONAL INFORMATION AND DECLARATIONS**

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**Conflict of interest statement**

The authors declare there are no competing interests.

**Author Contributions**

- Changying Liu, Maode Yu and Aichun Zhao conceived and designed the experiments.
- Changying Liu, Jun Li, Panpan Zhu, Jian Yu, Jiamin Hou, and Chuanhong Wang performed most of the experiments.
- Changying Liu and Dingpei Long analyzed the data.
- Changying Liu and Aichun Zhao wrote the manuscript.
- All the authors read and approved the final manuscript.

**Data Availability**

The following information was supplied regarding data availability:

- The raw data has been supplied as Data S1.
Supplemental Information

Supplemental information for this article can be found online at

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FIGURE LEGENDS

Figure 1 Expression profiles of MnEIL genes in response to salt and drought stresses. Mulberry seedlings were subjected to salt [0.6 % (m/v) NaCl] and drought [20 % (m/v) PEG6000]. (A) MnEIL gene expression levels in roots under salt stress. (B) MnEIL gene expression levels in shoots under salt stress. (C) MnEIL gene expression levels in roots under drought stress. (D) MnEIL gene expression levels in shoots under drought stress. Data are means ± SEs (n=3). Means within a column with different letters are significantly different (P <0.05). Means within a column with the same letters are no significant different (P >0.05).

Figure 2 Stress tolerance analyses of wild type, MnEIL3ox, and ein3-1 eil1-1 Arabidopsis. (A) The growth of wild type, MnEIL3ox, and ein3-1 eil1-1 Arabidopsis plants under normal, salt and drought conditions. (B–E) The survival rates (B), MDA content (C), H2O2 content (D), and proline content (E) of wild type, MnEIL3ox, and ein3-1 eil1-1 Arabidopsis plants under normal and stress conditions. Data are means ± SEs (n=3). Means within a column with different letters are significantly different (P <0.05). Means within a column with the same letters are no significant different (P >0.05).

Figure 3 qRT-PCR analysis of changes in AtACS and AtACO genes in MnEIL3ox plants. Data are means ± SEs (n = 3). Significant differences (P < 0.05) are marked with asterisks. The gene expression in wild type was set as 1.

Figure 4 In vivo interactions of MnEIL3 with ethylene biosynthetic genes promoters. (A) GUS staining of the injected strawberry fruit. (B) The detection of GUS activities. Data are means ± SEs (n=3). Significant differences (P <0.05) are marked with asterisks.

Figure 5 Analyses of promoter activities and expression levels of MnACS1 and MaACS3 genes in response to salt and drought treatments. (A) Histochemical GUS staining of the MnACS1pro::GUS and MnACS3pro::GUS transgenic Arabidopsis under salt [200 mM NaCl] and drought (200 mM mannitol) treatments. Scale bar, 2 mm. (B) The expression level of MnACS1 in response to salt treatment. (C) The expression level of MnACS1 in response to drought treatment. (D) The expression level of MnACS3 in response to salt treatment. (E) The
expression level of *MnACS3* in response to drought treatment. Data are means ± SEs (n=3).

Means within a column with different letters are significantly different ($P < 0.05$). Means within a column with the same letters are no significant different ($P > 0.05$).

**Figure 6** A possible model of *MnEIL3* in responses to abiotic stresses.
Expression profiles of *MnEIL* genes in response to salt and drought stresses. Mulberry seedlings were subjected to salt [0.6 % (m/v) NaCl] and drought [20 % (m/v) PEG6000]. (A) *MnEIL* gene expression levels in roots under salt stress. (B) *MnEIL* gene expression levels in shoots under salt stress. (C) *MnEIL* gene expression levels in roots under drought stress. (D) *MnEIL* gene expression levels in shoots under drought stress. Data are means ± SEs (n=3). Means within a column with different letters are significantly different (*P* <0.05). Means within a column with the same letters are no significant different (*P* >0.05).
Figure 2

Stress tolerance analyses of wild type, *MnEIL3ox*, and *ein3-1 eil1-1* Arabidopsis. (A) The growth of wild type, *MnEIL3ox*, and *ein3-1 eil1-1* Arabidopsis plants under normal, salt and drought conditions. (B–E) The survival rates (B), MDA content (C), H$_2$O$_2$ content (D), and proline content (E) of wild type, *MnEIL3ox*, and *ein3-1 eil1-1* Arabidopsis plants under normal and stress conditions. Data are means ± SEs (n=3). Means within a column with different letters are significantly different (P< 0.05). Means within a column with the same letters are no significant different (P >0.05).
qRT-PCR analysis of changes in AtACS and AtACO genes in MnEIL3ox plants. Data are means ± SEs (n = 3). Significant differences (P < 0.05) are marked with asterisks. The gene expression in wild type was set as 1.
Figure 4

In vivo interactions of MnEIL3 with ethylene biosythetic genes promoters. (A) GUS staining of the injected strawberry fruit. (B) The detection of GUS activities. Data are means ± SEs (n=3). Significant differences (P <0.05) are marked with asterisks.
A  

| Empty vector | MnACO1 | MnACO2 | MnACS1 | MnACS3 |
|---------------|-------|-------|-------|-------|
| ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) |
| MnEL1 | MnACO1 | MnACO2 | MnACS1 | MnACS3 |
| ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) | ![Image](image10.png) |
| MnEL2 | MnACO1 | MnACO2 | MnACS1 | MnACS3 |
| ![Image](image11.png) | ![Image](image12.png) | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) |
| MnEL3 | MnACO1 | MnACO2 | MnACS1 | MnACS3 |
| ![Image](image16.png) | ![Image](image17.png) | ![Image](image18.png) | ![Image](image19.png) | ![Image](image20.png) |

B  

| Treatment | Relatively GUS Activity (nmol/ml/min/mg) |
|-----------|---------------------------------------|
| MnACO1    | ![Image](image21.png) |
| MnEL1+MnACO1 | ![Image](image22.png) |
| MnEL2+MnACO2 | ![Image](image23.png) |
| MnEL3+MnACO3 | ![Image](image24.png) |
| MnACS1    | ![Image](image25.png) |
| MnELS+MnACS2 | ![Image](image26.png) |
| MnACS3    | ![Image](image27.png) |

* Indicates statistical significance.
Figure 5

Analyses of promoter activities and expression levels of *MnACS1* and *MaACS3* genes in response to salt and drought treatments. (A) Histochemical GUS staining of the MnACS1pro::GUS and MnACS3pro::GUS transgenic *Arabidopsis* under salt [200 mM NaCl] and drought (200 mM mannitol) treatments. Scale bar, 2 mm. (B) The expression level of *MnACS1* in response to salt treatment. (C) The expression level of *MnACS1* in response to drought treatment. (D) The expression level of *MnACS3* in response to salt treatment. (E) The expression level of *MnACS3* in response to drought treatment. Data are means ± SEs (n=3). Means within a column with different letters are significantly different (*P* <0.05). Means within a column with the same letters are no significant different (*P* >0.05).
A possible model of $\textit{MnEIL3}$ in responses to abiotic stresses.