Characterization and functional analysis of transcription factor ZmEIL1 in maize

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Abstract

As key nuclear transcription factors, the ethylene-insensitive3/EIN3-like (EIN3/EIL) proteins play important roles in ethylene signal transduction pathway in various plants. In order to better understand the role of EIN3/EILs, one EIN3-like gene (designated ZmEIL1) was isolated from maize (Zea mays L.). The full-length cDNA of ZmEIL1 was 1999 bp in length and encoded 647 amino acids. Sequence comparison of ZmEIL1 protein with other EIN3/EILs proteins revealed high conservation of five α-helices that could form a V-shaped cleft in a 3-D model, just like AtEIL3 in Arabidopsis thaliana. This protein showed transcriptional activation and activation domain located on the 507 - 647 amino acids in yeast. Furthermore, ZmEIL1 could interact with ZmERF1 in the yeast systems, which was downstream response factor in ethylene signal transduction pathway. Its mRNA could be highly induced in maize seedlings by ethephon and 1-methylcyclopropene treatments. Meanwhile, ZmEIL1 showed relatively high expression at 20 d after pollination in maize kernel. These results show that ZmEIL1 played an important role in the growth and development by participating in ethylene signalling pathway in maize.

Introduction

The gaseous phytohormone ethylene affects a wide range of physiological and morphological traits in plants, such as seed germination, growth, leaf and petal abscission, fruit ripening, and organ senescence (Guo and Ecker 2004). In addition, the involvement of ethylene in responses of plants to various biotic and abiotic stresses has been highlighted. In the past two decades, the ethylene signal transduction pathway was established based on the identification of ethylene-response mutants in Arabidopsis. Ethylene, which is perceived by a family of membrane-associated receptors, stimulates the downstream signalling pathway mediated by ethylene insensitive 2 (EIN2). Ethylene signalling downstream of EIN2 is mediated by EIN3, a plant-specific transcription factor (Cao et al. 2007).

In recent years, EIN3/EIN3-like (EIL) proteins have received attention. As a key nuclear transcription factor in the ethylene signalling pathway, EIN3/EIL is a short-lived protein that is degraded by an ubiquitin/26S proteasome pathway (An et al. 2010). There are five EIN3-like homologs (EIL1 to EIL5) in the Arabidopsis, among which EIL1 is closely related to EIN3. EIN3 in Arabidopsis encodes a protein of 628 amino acid residues with DNA binding domain (80 - 359 aa), dimerization domain (113 - 257 aa), and C terminal. EIN3/EIL genes are involved in a regulatory cascade and trigger ethylene responses, mainly via the regulation of ethylene response factor (ERF) genes, which belong to the AP2/ERF family

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Abbreviations: AD - activation domain; BD - DNA binding domain; DAP - days after pollination; EIN3/EIL - ethylene-insensitive3/EIN3-like; ERF - ethylene response factor; ET - ethephon; 1-MCP - 1-methylcyclopropene; ORF - open reading frame; qPCR - quantitative PCR; RT-PCR - reverse transcription PCR; SD - synthetic dextrose.

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Materials and methods

Isolation and bioinformation analysis: A BLASTP search was performed using the amino acid sequence of the EIN3 from Oryza sativa var. japonica (GenBank accession No. BAB78462) in NCBI, and a tentative consensus sequence of NP_001152035 located on chromosome 1 was identified in maize. Based on the nucleotide sequence of NP_001152035, primers (ZmEIL1-F and ZmEIL1-R) covering the open reading frame (ORF) were designed to obtain the cDNA sequence in maize inbred line N04 (Table 1).

The reverse transcription (RT)-PCR (Tiangen, Beijing, China) was performed according to the following procedures: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 2 min, and a final extension at 72 °C for 10 min. Together with the deduced ZmEIL1 amino acid sequence, the full length nucleotide sequence of ZmEIL1 was then identified and submitted to NCBI (acc. No. KT156845).

Multiple alignment of the ZmEIL1 sequence was accomplished with BioXM 2.6 software. The phylogenetic tree based on the result of ClustalW protein alignments was constructed by the software of Mega 4.0 using the neighbor-joining method (Tamura et al. 2007). Homology modeling was performed with the SWISS-MODEL program on the ExPaSy web server (http://www.expasy.org/) (Arnold et al. 2006).

Plants and stress treatments: Zea mays L. inbred line N04 was planted at the Scientific Research and Education Center of the Henan Agricultural University in Zhengzhou, Henan, China, in 2014. Ears were harvested at 10, 20, and 33 d after pollination (DAP). Grains were isolated from the middle part of the ears. At each time point, the samples were collected from at least three ears and pooled, with three replications. To investigate the effect of ethylene on the expression of ZmEIL1, the maize seedlings were grown in hydroponic culture with half-strength Murashige and Skoog (MS) nutrient solution in a growth chamber at a temperature of 26 °C, a 16-h photoperiod, an irradiance of 200 µmol m⁻² s⁻¹, and a relative humidity of 60 - 70 %.

Real-time quantitative (q) PCR: Total RNA was isolated and digested with RNase-free DNase I (TaKaRa, Dalian, China) to remove the residual genomic DNA. The first-strand cDNA was synthesized from 2 µg of DNA-free RNA using a PrimeScript RT reagent kit (TaKaRa). The qPCR reactions were performed as follows: 95 °C for 10 min, then 40 cycles of 95 °C for 10 s, 58 °C for 35 s, and 72 °C for 30 s, and a final extension at 42 °C for 10 min. ZmActin was used as the endogenous control. The expression level was calculated by the comparative CT method (Livak and Schmittgen 2001). All reactions were performed in triplicate for each of the three biological replicates.

Transcriptional activity analysis: The yeast two-hybrid system was used to analyze ZmEIL1 transcription. Truncated versions of ZmEIL1 were amplified by PCR and cloned between the Smal and BamHI sites of pGBKT7. The fused proteins were as follows: ZmEIL1-N1 (1-647 aa), ZmEIL1-N2 (1-507 aa), ZmEIL1-N3 (1-423 aa), ZmEIL1-N4 (1-308 aa), ZmEIL1-N5 (1-158 aa), ZmEIL1-C6 (423-647 aa), ZmEIL1-C7 (336-647 aa) (Fig. 4f). The primers used are listed in Table 1 Suppl. All recombinant plasmids were introduced into the yeast strain Y2HGold, and then the yeast were grown at 30 °C for 3 d. According to the manufacturer protocol (Clontech, CA, USA), the growth status of the transformed yeast cells were compared on Maize is one of major crops and model plants for genetic studies. EIN3/EILs, as key components in the ethylene signalling pathway, have still not been known in maize. Here, ZmEIL1 was isolated successfully from the maize inbred line N04. Furthermore, the expression of ZmEIL1 in response to ethephon (ET) and 1-methylcyclopene (1-MCP) were monitored to explore the role of the gene during the exogenous hormone treatment. Moreover, the relationship between ZmERF1 and ZmEIL1 proteins were investigated. These results would lay a good foundation for further study on the molecular mechanism of the ethylene signalling pathway in maize.

(Fujimoto et al. 2000). The AP2/ERF transcription factors have been shown to act as activators or repressors of additional downstream ethylene-responsive genes during adaptation to abiotic stresses in plants (Huanga et al. 2010, Wang et al. 2013). Moreover, EIN3/EIL1 could also take part in regulating gene expression of other signalling pathways, for instance, photomorphogenesis (Zhong et al. 2009, 2012), auxin biosynthesis and transport (He et al. 2011), cytokinin signalling reactions (Shi et al. 2010), salicylic acid synthesis (Chen et al. 2009), salt stress (Zhang et al. 2011), and iron metabolism (Lingam et al. 2011).
selective synthetic dextrose (SD) plates SD/-trp/X-a-Gal/AbA* and SD/-trp-his-ade (250 ng dm⁻³).

Interaction between ZmERF1 and ZmEIL1 proteins:
The pGADT7 and pGBK7 vectors contained a GAL4 activation domain (AD) and DNA binding domain (BD), respectively. ZmERF1 had been cloned in our previous studies (Shi et al. 2015) and its transcription activity was detected like that of ZmEIL1 (Fig. 5A). ZmEIL1-N2 was chosen and cloned into the pGADT7 vectors to generate the construct pGADT7-EIL1 with a fusion protein of a GAL4 AD and ZmEIL1. To determine whether these two proteins could interact in vitro, the empty pGBK7 vectors, pGADT7-ZmEIL1 vectors, pGBK7-ZmERF1 vectors, and pGADT7-ZmEIL1 vectors were cotransformed into Y2HGold competent cells according

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**ZmEIL1**

**AtEIL3**

Fig. 1. A - Nucleotide and deduced amino acid sequences of ZmEIL1 from maize inbred line N04. Nuclear localization sequence was shown with shadow and five α-helices regions were underlined; B - 3-D structure of ZmEIL1 fragment (213 - 340 aa) and AtEIL3. Five α-helices regions are marked.
ANALYSIS OF TRANSCRIPTION FACTOR ZmEIL1

Results

A full-length cDNA was identified as ZmEIL1 based on the EIN3 gene from Oryza sativa. To verify the result, specific primers were used to amplify the ORF fragment of ZmEIL1 via RT-PCR (Fig. 1 Suppl.). DNA sequence analysis showed the length of ZmEIL1 of 1999 bp with a 1941 bp ORF encoding 647 amino acids (Fig. 1A). To reveal the protein character of ZmEIL1, a 3-D model of the ZmEIL1 protein was established by SWISS-MODEL using the homology-modelling method. The template for modelling was the crystal structure of the AtEIL3 protein (PDB: 1wijA; Yamasaki et al. 2005). A 3-D structure of the ZmEIL1 protein fragment (213-340 aa) containing five α-helices could also form a V-shaped cleft (Fig. 1B).

The lysine 245 (K) located in α-helix 3, the key amino acid residue for the DNA-binding and signal transduction activities, was identified in ZmEIL1 protein. These

Fig. 2. A - Amino acid alignment of ZmEIL1 with homologous proteins from other plants. Black triangle indicates the key lysine. Red frame indicates five small clusters of basic amino acids. Green frame indicates the highly acidic amino acids. The amino acid sequences are as follows: NtEIL (AAP03997.1), AtEIL (AEE76421.1), SIEIL (AF328784_1), and ZmEIL1.

B - Phylogenetic analysis of plant EILs proteins. The protein sequences in the analysis are NtEIL (AAP03997.1), AtEIN3 (AEE76421), SIEIL (AAK58857), VrEIL2 (AAL76271), OsEIL2 (BAB78462), GaEIL (KHG09663), OsEIL1 (BAD10248), PtEIL (XP_002310961), AtEIL2 (NP_197611), AtEIL1 (NP_180273), and AtEIL3 (NP_177514).

plates DDO/X (SD/-Leu-/Trp/X-a-Gal) and QDO/X/A (SD/-Ade/-His/-Leu/-Trp/ X-a-Gal /Ab4*) at 30 °C for 3 d.

to the manufacturer’s instructions (Clontech). The transformed yeast cells were cultured on selective SD plates DDO/X (SD/-Leu-/Trp/X-a-Gal) and QDO/X/A (SD/-Ade/-His/-Leu/-Trp/ X-a-Gal /Ab4*) at 30 °C for 3 d.
sequence alignments revealed that it had high homology in the N-terminus with AtEIN3, including five small clusters of basic amino acids (Fig. 2A).

Further, a phylogenetic tree was constructed based on the amino acid sequences in plants to understand the evolutionary relationships with ZmEIL1. The result revealed that EIN3/EILs could derive from a common ancestor during evolutionary processes (Fig. 2B). ZmEIL1 was grouped into a cluster with monocotyledons belonging to the grass family and paralleling evolutionary relationships among them.

Real time qPCR analysis was used to reveal ZmEIL1 expression patterns in the endosperm and pericarp at 10, 20, and 33 DAP. The results showed that the expression of ZmEIL1 was lower at 10 DAP and 33 DAP compared to that at 20 DAP. ZmEIL1 at 20 DAP and 33 DAP in the pericarp had higher expression than that in the endosperm (Fig. 3A). This shows that ZmEIL1 could play a role in kernel formation and development in maize.

Under ET treatment, ZmEIL1 expression in leaves was 9-fold higher than that of the control at 9 h after treatment, and then gradually decreased to the level in the control plants after 24 h (Fig. 3B). The expression pattern was similar in roots and in leaves where ZmEIL1 was significantly up-regulated at 6 h and then gradually decreased (Fig. 3D). Under 1-MCP stress, the expression of ZmEIL1 in leaves was down-regulated during the 24-h period except at 6 h (Fig. 3C). However, ZmEIL1 expression in roots was induced up to 10-fold after 6 h under 1-MCP stress and then decreased (Fig. 3E). These observations suggested that ZmEIL1 was regulated by the concentration of ethylene, and likely to be involved in the ethylene signaling pathway.

Yeast two-hybrid system was applied to examine whether ZmEIL1 had potential transactivation activity. The full length of ZmEIL1 was fused to GAL4 DNA-binding domain of pGBK7 and then it was transformed into the yeast strain Y2HGold. Yeast cells transformed with ZmEIL1-N1 could grow well on SD/-trp/X-a-Gal/AbA* and showed blue coloration. Further, they could grow well on SD/-trp-his-ade medium that indicated ZmEIL1 was a transcriptional activator, which might regulate its downstream genes (Fig. 4B). To find motif with the activate function, truncated versions of ZmEIL1 were also fused to the pGBK7 (Fig. 4A). In yeast strains, the transformants harboring all recombinant plasmids could grow on SD/-trp/X-a-Gal/AbA*, but only yeast cells with ZmEIL1-C6 and ZmEIL1-C7 could turn blue and grow on SD/-trp-his-ade medium (Fig. 4B). Together these results indicated that the transcriptional activation domain of ZmEIL1 is located on the 507-647 amino acids at the end of the C-terminal region.

The EIN3/EIL proteins generally exert their function by binding with the target promoter elements, such as ERF1. In this study, the interaction between ZmEIL1 and ZmERF1 proteins was tested using yeast two-hybrid assay (Fig. 5B). The yeast cells carrying ZmERF1-1 and ZmERF1-C7 grew well on SD/-trp/X-a-Gal/AbA* and SD/-trp-his-ade and turned blue when subjected to X-a-Gal. These results confirmed that the activating domain of ZmERF1 was located 154-229 aa (Fig. 5A), and therefore ZmERF1-N2 was chosen for future experiment.

Fig. 3. The expression profile of ZmEIL1 was detected using real time qPCR. A - expression of ZmEIL1 in pericarp and endosperm; B, D - expression of ZmEIL1 in four-leaf stage (V4) seedlings treated with ethephon (ET), and C, E - in leaves and roots treated with 1-MCP. Means ± SE, n = 3, * and ** indicate significant differences from the control at P ≤ 0.05 and P ≤ 0.01, respectively.
The ADZmEIL1 and BKZmERF1 vectors were co-transformed into yeast strains Y2HGold. The yeast cells growing well on the DDO/X SD plate showed that co-transformation test was performed successfully. And the yeast cells growing well and turning blue on the QDO/X/A medium showed that the two proteins could interact in vitro. In the controlled trial, yeast cells harbouring pGBK7 and ADZmERF1 vectors could grow well on the DDO/X plates, but do not grow on the QDO/X/A (Fig. 5C). These result suggested that there might exist interaction between ZmERF1 and ZmEIL1.

Fig. 4. A - Schematic overview of the various deletion mutants of ZmEIL1 that were used to analyze transcription-stimulating activity in yeast. B - The ZmEIL1 and its various deletion fragments fused with the GAL4 DB expression vector were transformed into yeast strain Y2HGold. The transformants were selected on SDO/-trp/X/A and SDO/-trp-his-ade media at 30 °C for 3 d. Yeast transformants were streaked on the same media and were grown at 30 °C for 3 d.

Discussion

EIN3/EIL proteins belong to a small family which are positive regulators at downstream position of ethylene signal transduction pathway (Chao et al. 1997, Mao et al. 2006). In this study, ZmEIL1 was cloned and analyzed. ZmEIL1 owned highly conserved amino acid sequences and the similar functional domain compared with other members of the family. Specifically, ZmEIL1 showed high homology with its counterpart (OsEIL2) in rice and was grouped into the same cluster. ZmEIL1 was localized in the nucleus via prediction of subcellular localization using SoftBerry (http://linux1.softberry.com/) (Fig. 2 Suppl.). These results were consistent with EIN3/EILs being nuclear transcription factors.

In Campanula species, EIN3/EIL mRNA accumulation is not affected by ethylene treatment, and it is constitutively expressed during flower and fruit development, and fruit ripening (Jensen et al. 2016). However, other evidences also show that some EIN3/EIL mRNAs could be regulated at the transcriptional level, based on the fact that exogenous ethylene could regulate the accumulation of EIN3/EILs mRNA (De Paepe et al. 2004, Parra-Lobato and Gomez-Jimenez 2011). In our study, the expression of ZmEIL1 after ethephon application increased to a peak values and then decreased (Fig. 3B,D). Similarly, in Dianthus caryophyllus flowers treated with ethylene, the amount of mRNA of DC-EIL3 remains elevated from 4 to 12 h and afterward decreases (Iordachescu and Verlinden 2005). However, there were many differences in the expression pattern in leaves and roots under 1-MCP treatment. It was noteworthy that the accumulation of ZmEIL1 mRNA in leaves was down-regulated under 1-MCP treatment, but it was up-regulated in roots (Fig. 3C,E).

EILs genes are involved in transcriptional regulation
of ripening-related genes and also in the regulation of kiwifruit fruit-ripening (Yin et al. 2010). LeEIL genes could modulate ethylene response and fruit development in tomato (Tieman et al. 2001). EIN3/EIL1 could act as a hub in plant signalling networks that sensed a broad range of stimuli and integrated these stimuli to affect plant growth, development, and stress resistance. The expression of ZmEIL1 was different at different stages of kernel development (Fig. 3A). Therefore, ZmEIL1 could play important roles in regulating kernel development and maturation in maize.

A model for the ethylene signal transduction had been established in Arabidopsis. At least, five gene families including EIN3/EILs and ERFs were involved in this pathway (Kendrick and Chang 2008). According to Solano et al. (1998), nuclear proteins EIN3 and ERF1 acted sequentially in a cascade of transcriptional regulation initiated by ethylene in Arabidopsis. The pathway analysis had been extended to fruit species, but less in crops (Yin et al. 2010). In the previous study, an ethylene response factor, ZmERF1, has been isolated and characterized from maize, the homologs gene to the Arabidopsis AtERF1 (Shi et al. 2015). Meanwhile, ZmERF1 and ZmEIL1 proteins were capable of functioning as transcriptional activators (Fig. 4B, 5B). In the transgenic Arabidopsis plants, high heterologous expression of ZmERF1, AtEIN3, and AtEIL1 was

![Fig. 5. Interaction between ZmERF1 and ZmEIL1 proteins. A - Schematic overview various deletion mutants of ZmERF1. B - The ZmERF1 and its deletion constructs fused with the GAL4 DB expression vector were transformed into yeast strain Y2HGold to investigate transcription activity, respectively. The transformants were selected on SDO/-trp/X/A and SDO/-trp-his-ade media at 30 °C for 3 d. C - Interaction between ZmEIL1 and ZmERF1 in yeast two-hybrid assay. The ADZmEIL1 and BKZmERF1 vectors were co-transformed into yeast strain Y2HGold. The transformants were selected on DDO/X and QDO/A/X media at 30 °C for 3 d.](image-url)
constitutive (Fig. 3 Suppl.). ERF genes could be necessary for EIN3/EIL expression. Thus, it would be intriguing to identify the interaction between ZmEIL1 and ZmERF1 in vitro. Yeast two-hybrid assay also confirmed the interaction between them (Fig. 5C). Within the pathway, EIN3/EILs and ERFS served as TFs that could bind conserved motifs in promoter regions of target genes, thus activating the ethylene signal to the level of target gene transcription (Solan et al. 1998). EIN3/EIL1/EIL2 could regulate the expression of ERF1 by binding to the promoter elements of Arabidopsis (Yin et al. 2010). So we speculated that ZmEIL1 not only could regulate ZmERF1, but could be regulated by ZmERF1 protein.

In conclusion, the full-length cDNA of ZmERF1 had been successfully cloned from maize. ZmEIL1 could interact with ZmERF1 in yeast two-hybrid systems. Meanwhile, we analyzed expression patterns of ZmEIL1 of maize seedling treated with ET and 1-MCP in endosperm and pericarp at different developmental stages. This study lays the foundation for further exploring the role of ZmEIL1 in ethylene signalling pathway in maize. However, if we want to obtain a better comprehensive picture of ethylene signalling pathway in maize, a lot of work is still needed to determine more ZmEIL1 functions.

References


