

EAR motif mutation of rice OsERF3 alters the regulation of ethylene biosynthesis and drought tolerance

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Abstract OsERF3 is a transcriptional repressor with an ethylene-responsive element-binding factor-associated amphiphilic repression (EAR) motif (F/LDLNxxP), which transcriptionally represses the ethylene emission and drought tolerance in rice. However, its molecular mechanism to explore repression function remains unknown. Here, we first revealed that the expression of *OsERF3* was induced by drought, salt, ACC and ABA treatment. In addition, it showed a higher expression level in the root and sheath than that in the leaf. Then, we generated transgenic rice over-expressing full-length *OsERF3* (OE) and its mutation of EAR motif with the A₆₈₀/C substitution (mEAR), respectively. The physiological analyses showed that mEAR lines showed better drought tolerance and more ethylene emission compared with those of OE lines and wild type plants. Consistent with our previous research, the expression of ethylene synthesis genes, including *ACO2*, *ACS2*, and *ACS6* was down-regulated in OE lines. However, the repression of

OsERF3 was eliminated in mEAR lines. Specifically, *ACS2* was up-regulated in mEAR lines compared with that in OE lines and WT plants, suggesting that the Leu/Ala substitution within the EAR motif resulted in loss of repression of OsERF3. Thus, our data reveal that the EAR motif is required for OsERF3 to transcriptionally regulate the ethylene synthesis and drought tolerance in rice, providing new insight to the roles of ethylene-response factor proteins in regulating ethylene biosynthesis and stress response.

Keywords ERF protein · Ethylene function · Drought response · Rice · Transcriptional repression

ERF	Ethylene-response factor
EAR	Ethylene-responsive element-binding factor-associated amphiphilic repression
ACC	1-Aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
GUS	Beta-glucuronidase
ABA	Absciscic acid
JA	Jasmonic acid
IAA	Indole-3-acetic acid
GA	Gibberellic acid

H. Zhang and J. Zhang contributed equally to this work.

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Introduction

In plants, drought stress induces a series of physiological and biochemical responses, including stomatal closure, repression of cell growth and photosynthesis, activation of respiration, and accumulation of stress-related osmolytes and proteins (Shinozaki and Yamaguchi-Shinozaki 2007). At the molecular level, many genes with diverse functions

were induced or repressed by drought (Xu et al. 2010; Yaish et al. 2010; Zhou et al. 2010). Ethylene is an important phytohormone, which triggers a wide range of physiological reaction involved in plant developmental processes and environmental stresses (Bleecker and Kende 2000). For instance, NTHK1 is the second class ethylene receptor of tobacco, whose overexpression resulted in the decreased tolerance to salt in rice, while extraneous ACC treatment can repress this sensitivity (Cao et al. 2007; Chen et al. 2009). Our previous investigation showed that ethylene application could enhance drought tolerance in rice (Wan et al. 2011). The synthesis of ethylene is induced by different environmental stimuli and involved in plant response to drought, wound, and defense against pathogens (Kar 2011). In addition, the transcriptional regulation of ethylene biosynthesis-related genes has been reported as a key process in affecting ethylene production, which further triggers the expression of stress-related genes and plant adaptation to stresses (Wang et al. 2002; Zhang and Huang 2010; Wan et al. 2011).

The ethylene-response factor (ERF) proteins play key roles in plant development and stress response by regulating the expression of numerous genes in different plant species (van der Fits and Memelink 2001; Gu et al. 2002). In rice, more ERF proteins were reported to play multiple roles in development processes and stress response (Fukao and Bailey-Serres 2008). For example, OsEATB affects rice tillering and panicle branching through down-regulating the expression of gibberellin biosynthetic genes (Qi et al. 2011). Auxin-induced *CRL5* controls crown root initiation by repressing cytokinin signaling (Kitomi et al. 2011). Moreover, OsDERF1 modulates drought response through transcriptionally controlling ethylene synthesis (Wan et al. 2011). OsERF922 is involved in the cross-talk between biotic and abiotic stresses (Liu et al. 2012). Sub1A participates in rice submergence and drought tolerance through mediating different hormone pathways (Xu et al. 2006; Fukao and Bailey-Serres 2008; Fukao et al. 2011). OsAP2-39 is associated with the rice yield and drought tolerance through regulating the GA/ABA balance (Yaish et al. 2010).

The ethylene-responsive element-binding factor-associated amphiphilic repression (EAR) motif (with a consensus of L/FDLNL/FXP) was a kind of transcriptional repression motif with diverse biological functions, which was identified from multiple protein families including class II ERFs, AUX/IAA proteins, JAZ proteins, and TFII-type zinc finger proteins (Kagale and Rozwadowski 2011). For example, JAZ8-mediated repression depends on an L × L × L-type EAR motif that binds the corepressor TOPLESS and represses transcriptional activation, suggesting that EAR motif-containing JAZ proteins repress JA responses by recruiting TPL/TPRs to transcription factors (Shyu et al. 2012). Class II ERF proteins contain a

conserved EAR repressor motif in their C terminals (Fujimoto et al. 2000). In plants, a series of ERF proteins containing EAR-motif such as NtERF3, AtERF3, AtERF4, AtERF7, and AtERF11 were reported to play important roles in modulating hormone balance and various stress signaling (Ohta et al. 2001). For example, *AtERF11* plays a key role in regulating ethylene biosynthesis by negatively tuning the expression of *AtACS2/5* in Arabidopsis (Li et al. 2011). AtERF7 functions as a transcriptional repressor involved in regulating ABA responses and drought sensitivity by interacting with PKS3 and AtSin3 (Song et al. 2005). However, ectopic expression of an EAR motif deletion mutant of *SlERF3* resulted in the increased tolerance to salt stress and *Ralstonia solanacearum* in tomato by regulating the expression of several pathogenesis-related genes (Pan et al. 2010).

OsERF3 (Os01g0797600) belongs to class II ERFs with a conserved EAR-motif (DLNRPPP) at the C terminal (Nakano et al. 2006). In rice, OsERF3 participated in the resistance to disease and the herbivore-induced defense responses via mediating different hormone pathways (Lu et al. 2011). We also found that OsERF3 negatively affected ethylene production and drought tolerance in rice (Wan et al. 2011). However, little is known about whether its EAR motif is involved in the regulation of ethylene synthesis and drought response. Here, we generated transgenic rice overexpressing full-length (OE) and the mutation of *OsERF3* with the A₆₈₀/C substitution (mEAR), respectively. The phenotypic analyses suggested that mEAR lines showed better drought tolerance and more ethylene emission than those of OE lines and wild type (WT) plants. At the transcriptional level, the substitution of Leu/Ala disrupted the repression of *ACO2* and *ACS6* by OsERF3 in mEAR lines. Moreover, *ACS2* was significantly up-regulated in mEAR lines compared with that in WT plants. Taken together, our results indicate that the EAR motif is required for OsERF3 to repress the expression of ethylene biosynthesis genes, deepening our understanding of the regulatory function of ERF proteins involved in ethylene biosynthesis and stress response.

Materials and methods

Plant material and drought stress treatment

Rice (*Oryza sativa* L.) seeds (preserved by our lab) of wild type (WT) Nipponbare and transgenic lines were used in the drought stress treatment. After germination at 30 °C for 2 days, the germinated seeds were transplanted on the sandy soil in the greenhouse at 26 °C and 16 h light/8 h dark. The 2-week-old seedlings were exposed to successive drought by withholding water. For the control, all of

seedlings were kept in normal growth conditions with water. When the seedlings began to wilt at the 6th day, the drought phenotypes among different lines were observed. After all seedlings showed varying degrees of stress symptoms at the 9th day, they were recovered normal condition with water for 7 days, the growing status were photographed and the survival rate was analyzed statistically through *t* test.

Generation of transgenic rice

The full length of *OsERF3* gene was obtained from the cDNA library using specific primers. The mutation of EAR motif of *OsERF3* (mEAR) was obtained by replacing the base of A₆₈₀ with C (Fig. 1a) through PCR amplifications using specific primers (Table S1). Then, the full-length cDNA of *OsERF3* and mEAR were inserted into pCAMBIA1307 modified from pCAMBIA1300, respectively. For the modification of pCAMBIA1307 vector (Fig. S1), we inserted a fragment including a CaMV 35S promoter and multiple cloning sites from vector pBluescript into the *Sst*I and *Kpn*I of pCAMBIA1300 vector. To generate the *P_{ERF3}*:GUS construct, the 1.0 kb promoter region upstream to start codon of *OsERF3* was amplified using specific primers (OsERF3P-F and OsERF3P-R) (Table S1). Then, the fragment digested with *Eco*RI and *Nco*I was cloned into the multiple cloning sites of pCAMBIA1391Z vector. All of the transgenic rice plants were generated through *Agrobacterium*-mediated transformation. T1 seedlings were examined by PCR amplifications at the DNA and RNA levels with specific primers (Table S1); T2 and T3 seeds were screened by germinating with the selection of 50 ng/l hygromycin.

Expression profiling by quantitative real-time PCR analysis

The materials used for gene expression analysis were cultured in the water for 7 days, and treated with 200 mM NaCl, 50 μ M ABA, or 50 μ M ACC for 12 h, respectively. For drought treatment, the seedlings were taken out from water and kept on filter paper for 12 h. Samples were collected at 0, 0.5, 1, 2, 4, 8, and 12 h after the treatment. Total RNAs of the collected samples were extracted using TRIZOL Reagent (Invitrogen, Beijing, China). Then, 1 μ l RNA was used to synthesize the first-strand cDNA with AMV Reverse Transcription System (Takara, Dalian, China) using oligo (dT) primers. For the expression of *OsERF3*, *ACO2*, *ACS2*, and *ACS6*, quantitative real-time PCR (qPCR) using SYBR Green I (Takara) was performed with their specific primers (Table S1). The expression of *OsActin1* (AB047313) was used as the internal control.

All experiments were repeated three times independently, and the average was calculated.

Quantification of ethylene biosynthesis

Ethylene emission by rice was measured with a gas chromatograph (Shimadzu, Tokyo, Japan) as described (Kim et al. 2003). Germinated seedlings were cultured in 50 ml glass vials (20 seedlings per vial) with MS culture medium. After recovery for 7 days, all the vials were sealed for 12 h. The ethylene emission was then determined by taking 100 μ l volumes with an air-tight syringe. Levels of ethylene in the vials were compared to those in the empty vials. The significant difference after calculated was analyzed statistically through *t* test.

Histochemical staining of GUS

For the histochemical staining of GUS in transgenic rice, the samples were incubated in the sodium phosphate buffer (pH 7.0) containing 0.1 % v/v Triton X-100 and 2 mM X-Gluc (Sangon, Shanghai, China) at 37 °C for 12 h. After rinsed with several changes of 70 % ethanol until tissue clears, the samples were photographed.

The OsERF3 sequence analysis

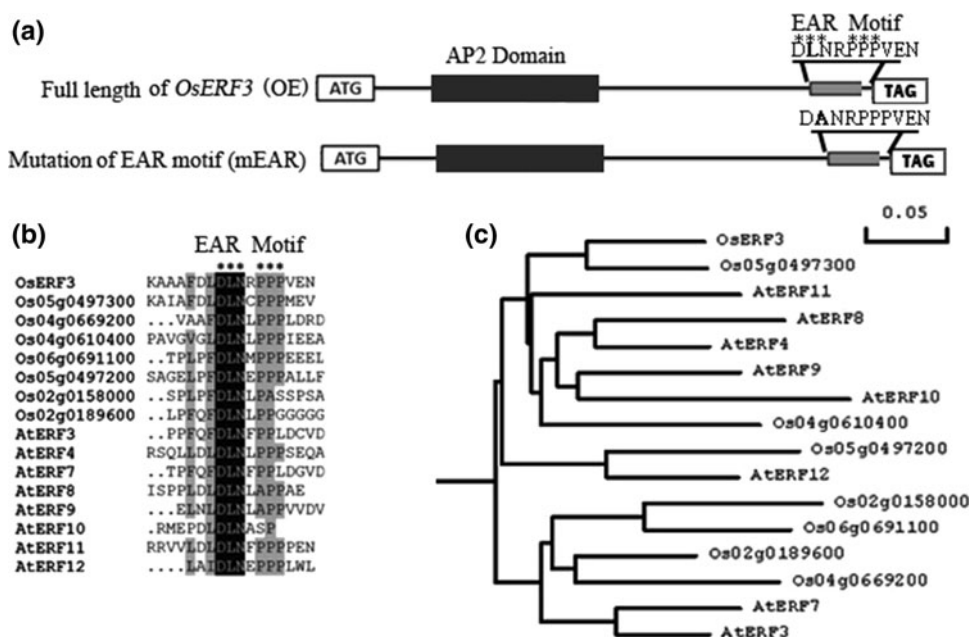
All sequences of the EAR-containing ERF proteins from rice and Arabidopsis were available at the National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov>). The nucleotide sequences and deduced amino acid sequences were aligned using the Multiple Sequence Alignment program of the DNAMAN software (Version 7.0, LynnonBiosoft, Quebec, Canada). A phylogenetic tree was also constructed from the alignment these sequences using DNAMAN software.

Results

The rice OsERF3 sequence analysis

The rice genome encodes for 163 putative AP2/ERF family proteins based on their conserved AP2 domain sequence (Sharoni et al. 2011). *OsERF3* encodes 235 amino acids and belongs to class II ERF proteins. Sequence alignments showed that OsERF3 has a conserved AP2 DNA-binding domain and an EAR motif (DLNRPPP), which were highly similar to the other class II ERF genes from Arabidopsis and rice. Especially, the three amino acids (DLN) were highly conserved in all candidate proteins (Fig. 1b, Fig. S2), suggesting they might play important roles in EAR

Fig. 1 Comparison of EAR motif and phylogenetic analysis of EAR motif containing ERF proteins from rice and Arabidopsis. **a** The diagram of the construct of mEAR by replacing the base of A_{680} with C, resulting in the change of Leu₂₂₇ to Ala. **b** Comparison of the amino acid sequence of EAR-motif. The asterisks mark the EAR motif. Amino acids identical in all proteins are shown in black; shaded represent at least three sequences are conserved. **c** The phylogenetic analysis was carried out and viewed by the DNAMAN software



motif-containing proteins. Analyses with phylogenetic tree of the protein sequences showed that *OsERF3* was grouped in a cluster with *Os05g0497300*, with identity of 67.2 %. These two proteins showed a close evolutionary relationship with *Os04g0610400*, *AtERF4*, *AtERF8*, and *AtERF11* (Fig. 1c), coinciding with the recent report on the syntenic relationships of rice ERF proteins (Rashid et al. 2012). Of these candidate genes, *AtERF11* (Li et al. 2011), *AtERF3* (Ohta et al. 2001), *AtERF4* (Yang et al. 2005), and *AtERF7* (Song et al. 2005) were proved to function as transcriptional repressors involved in modulating hormone response and stress tolerance. These analyses imply that *OsERF3* might be a transcriptional repressor and play roles in mediating stress response.

Expression pattern of *OsERF3* in response to various treatments and tissues

We investigated the expression pattern of *OsERF3* in response to various abiotic stresses and hormone treatments through qPCR analysis. The expression of *OsERF3* was highly induced by ABA at 1 h, and reached peak at 2 h. Similarly, it was rapidly inducible by ACC at 0.5 h and peaked at 2 h, and then it decreased to normal level at 8 h (Fig. 2a). Moreover, *OsERF3* was up-regulated by drought and peaked at 2 h. Similarly, *OsERF3* was quickly induced by high salinity and peaked at 0.5 h. These results indicate that *OsERF3* might be associated with ethylene- and ABA-related environmental stimuli in rice.

Moreover, we analyzed the potential *cis*-elements within the 1 kb region (1,040 bp) upstream to the start codon (Table S2). Our results showed that there were more

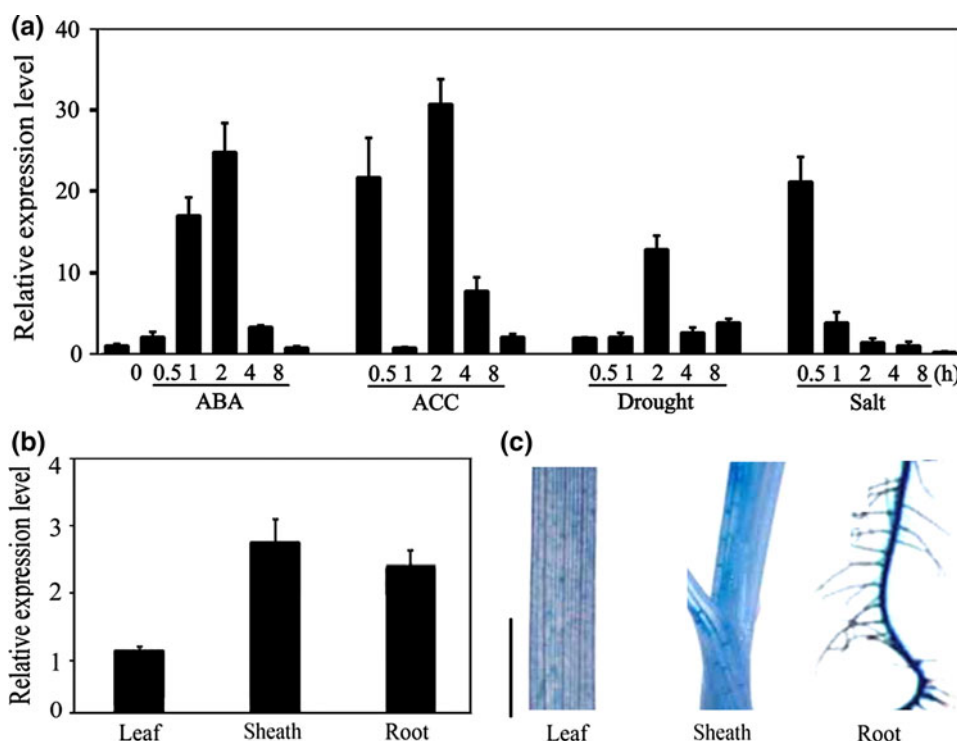
important elements involved in different hormones or stress signaling (including ethylene, ABA, Auxin and GA as well as dehydration, cold and light) and specific tissues. These data also support the molecular cues for the expression of *OsERF3* in response to different hormones and stress treatments.

We further analyzed its expression in different tissues. As shown in Fig. 2b, the expression of *OsERF3* in the root and sheath was two and three times higher than that in the leaf at the seedling phase, respectively. Moreover, the expression of GUS driven by the promoter of *OsERF3* in transgenic rice was analyzed. Consistently, the GUS staining was observed in all parts of seedlings, but it showed more intense in sheath and root than that in leaf (Fig. 2c), further confirming its expression in seedlings.

OsERF3 negatively regulates drought tolerance dependent on its EAR motif

We previously evidenced that *OsERF3* negatively regulated rice ethylene production and drought tolerance by repressing the expression ethylene synthesis-related genes (Wan et al. 2011). The putative transcriptional repressors rely on the EAR motif to explore their repression activity (Hamel et al. 2011). It has been reported that the Ala substitution within EAR motif resulted in partial or complete loss of repression by various EAR motif-containing proteins (Tiwari et al. 2004; Shyu et al. 2012). The Leu within EAR motif (DLNRP) of *OsERF3* was a highly conserved in this motif among related proteins from rice and Arabidopsis (Fig. 1b). To determine whether the EAR motif is required for *OsERF3* to implement its

Fig. 2 Expression of *OsERF3* in response to dehydration, hormones and at different tissues of seedling stage. The expression of *OsERF3* in 7-day-old seedlings in response to 200 mM NaCl, 50 μ M ABA, 50 μ M ACC, and drought (a), and in different tissues (b). Transcripts of *OsERF3* were referred to that of the control or the leaf. *OsActin1* was used as an internal control. Error bars (SD) were based on three independent experiments. c Analyses of GUS expression in the leaf (left), sheath (middle) and root (right) in transgenic rice harboring *P_{ERF3}::GUS*. Bar 2 cm



transcriptional repression, we carried out Ala-substituting mutagenesis by replacing the base of A_{680} with C through PCR amplification, which resulted in substituting the amino acid of Leu₂₂₇ with Ala (Fig. 1a). The transgenic rice over-expressing *OsERF3* (OE) or its mutant construct (mEAR) was generated through *Agrobacterium*-mediated transformation, respectively. After identification at the DNA and RNA levels (Fig. S3), two independent OE lines (OE-10, OE-12) and two mEAR lines (mEAR-1, mEAR-9) were selected for the further studies.

We first compared the drought tolerance among the transgenic lines (OE and mEAR) and wild type (WT) Nipponbare. As shown in Fig. 3a, most of the OE seedling leaves turned withered after the 2-week-old seedlings were exposed to successive drought by withholding water for 6 days, while WT and mEAR lines still grew well. When the seedlings of WT and OE lines exhibited serious stress symptom at the eighth day, mEAR lines showed mild stress symptom (Fig. 3a). After treated for 9 days, the resulting seedlings were recovered normal growth with watering for 7 days. The survival rates of seedlings in WT, OE lines, and mEAR lines were about 40, 19.5–28, and 43–49 %, respectively (Fig. 3b). Student's *t* test analyses showed that the differences among WT and two OE lines were significant ($P < 0.05$ or $P < 0.01$). Although there was no significant difference between WT and mEAR lines, the survival rate in mEAR lines was slight higher than that in WT plants. These findings suggest that the Leu₂₂₇/Ala

substitution results in the complete loss of negative regulation of drought tolerance by *OsERF3*. We also found that the OE lines showed drought-sensitive at the flowering stage (Fig. S4). Taken together, our research demonstrates that the EAR motif is necessary for *OsERF3* to negatively regulate drought tolerance in rice.

EAR motif is essential for *OsERF3* to repress the ethylene biosynthesis

We have previously verified that *OsERF3* could negatively control ethylene production (Wan et al. 2011). To investigate whether *OsERF3* affected the ethylene production through its EAR motif, we examined the ethylene emission in different transgenic lines. Our results showed that the ethylene emission in OE lines was about 70 % of that in WT. Student's *t* test analyses showed that there were significant differences in ethylene emission between WT and two OE lines (Fig. 4). However, the ethylene levels in two mEAR lines were higher than that in WT; and the difference between mEAR-1 and WT was significant at $P < 0.01$ level. These results revealed that the Leu₂₂₇/Ala substitution completely impaired *OsERF3* repression activity and subsequently enhanced the ethylene emission in mEAR lines, suggesting that EAR motif is required for *OsERF3* to negatively regulate ethylene production in rice.

It has been reported that series stress-related genes (Lu et al. 2011) and ethylene synthesis-related genes were

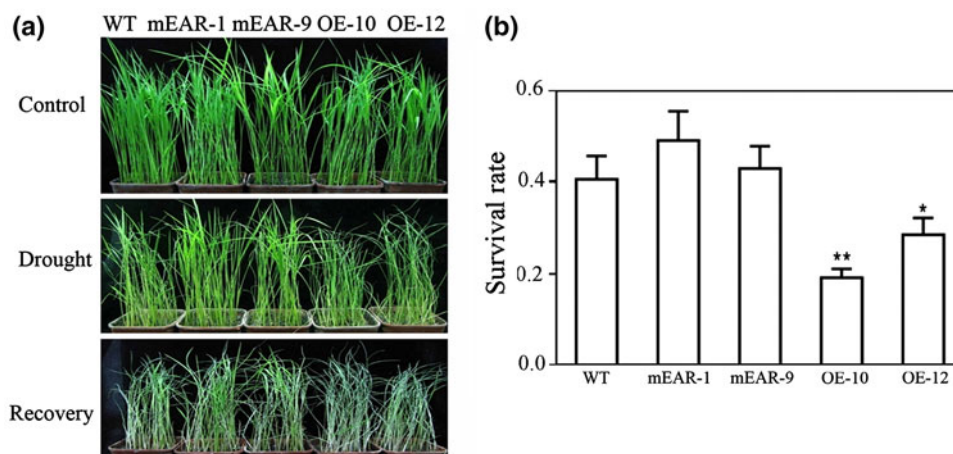


Fig. 3 Negative regulation of OsERF3 in the drought tolerance through its EAR motif. **a** Seedlings of OE, mEAR and Nipponbare (WT) plants subjected to drought stress. Control: 3-week-old seedlings growing under normal conditions. Drought: 2-week-old seedlings were subjected to successive drought for 9 days. Recovery: the seedlings were re-watered for 7 days after drought. **b** Survival

rates of the seedlings after recovery. The survival rate shows the percentage of recovered plants compared to the total tested plants. Each experiment was repeated three times with at least 40 seedlings for each sample. Student's *t* tests were conducted using the data in OE and mEAR lines and WT. * and ** indicate significant difference at $P < 0.05$ and $P < 0.01$ probability, respectively

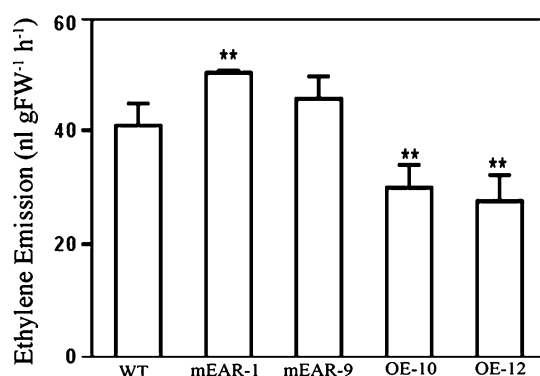


Fig. 4 Effects of OsERF3 on the ethylene production dependent on EAR motif. The data, average of three independent assays, show ethylene production in the transgenic lines and WT. The amount of ethylene per gram fresh weight seedling in an hour [nl gFW⁻¹ h⁻¹] is presented. Student's *t* tests were conducted using the data of transgenic seedlings compared to that of WT. * and ** indicate significant difference at $P < 0.05$ and $P < 0.01$ probability, respectively

the repression function of OsERF3, implying that the EAR motif is essential for OsERF3 to repress the expression of *ACO2*, *ACS2*, and *ACS6* in rice.

Discussion

In plants, drought stress induces a series of physiological and biochemical responses, which include stomatal closure, repression of cell growth and photosynthesis, and

regulated by OsERF3 in rice (Wan et al. 2011). In the present work, we focused on whether EAR motif is required for the repression of *OsACS2*, *OsACS6*, and *OsACO2* by OsERF3 in rice. The expression level of these genes in OE and mEAR lines was analyzed. As shown in Fig. 5, the expression level of *ACO2*, *ACS2*, and *ACS6* was decreased in OE lines. Interestingly, the expression of *ACS2* in mEAR lines was much higher than that in WT; the expression level of *ACO2* and *ACS6* in mEAR lines showed similar to that in WT. These results revealed that the mutation of Leu₂₂₇/Ala within the EAR motif disrupted

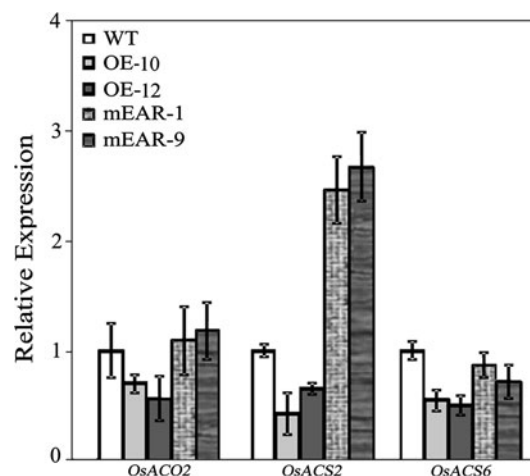


Fig. 5 Transcriptional repression of OsERF3 in the expression of ethylene biosynthesis-related genes via EAR motif. The expression of *OsACS2*, *OsACO2*, and *OsACO6* was detected by qPCR. The expression level of *OsActin1* was used as the internal control. The expression level of each gene in Nipponbare (WT) was standardized to 1. Error bars (SD) were based on three independent experiments

activation of respiration (Shinozaki and Yamaguchi-Shinozaki 2007). At the molecular level, some genes were induced or repressed in response to various stresses in different plant species (Xu et al. 2010; Yaish et al. 2010; Zhou et al. 2010). As the possible component of the ethylene signaling pathway, ERF proteins are responsible for modulating the transcription of ethylene-regulated genes, which play vital roles in mediating plant growth, development, and response to environmental stimuli (Pirrello et al. 2012; Rashid et al. 2012). In rice, a series of ERF proteins were proved to participate in rice growth and development by tuning different hormone signaling pathways (Fukao and Bailey-Serres 2008; Yaish et al. 2010; Kitomi et al. 2011; Qi et al. 2011), as well as response to biotic and abiotic stresses, such as drought (Yaish et al. 2010; Wan et al. 2011; Wang et al. 2012), salinity (Liu et al. 2012), disease (Liu et al. 2012), and submergence (Fukao et al. 2006; Jung et al. 2010). In the present research, our data reveal that the EAR motif is required for OsERF3 to transcriptionally regulate the ethylene synthesis and drought tolerance in rice, providing new insight to the roles of ERF proteins in regulating ethylene biosynthesis and stress response.

The EAR motif with a consensus of L/FDLNL/F(x)P is a small yet effective repression motif that is conserved in many plant transcriptional regulators involved in diverse biological functions (Kagale and Rozwadowski 2011). Stress-responsive MAPKs were involved in defense response and activated their target genes by degradation of EAR repressors in Arabidopsis (Hamel et al. 2011). A subgroup of JAZ proteins with EAR repression domains mediates transcriptional repression of jasmonate responses through direct recruiting corepressors to cognate transcription factors (Shyu et al. 2012), while NINJA function as negative regulators of jasmonate responses in Arabidopsis, which is mediated by a functional TPL-binding EAR repression motif (Pauwels et al. 2010). Class II ERF proteins play also key roles in diverse biological functions by negatively regulating genes involved in hormonal and stress signaling pathways. For example, AtERF7 negatively regulates drought tolerance by interacting with AtSin3, which might cause chromatin modification and repress the binding of activator with DNA through recruiting HDA19 (Chen and Wu 2010). AtERF4 acts as a transcriptional repressor capable of modulating ethylene and ABA responses (Yang et al. 2005). Importantly, AtERF11 functions as a key negative regulator for ABA-mediated control of ethylene synthesis through transcriptionally modulating the expression *ACS2/5* in Arabidopsis (Li et al. 2011). In addition, RAP2.1 functions as a negative transcriptional regulator in defense responses to environmental stresses through tuning the expression of stress-related genes in Arabidopsis (Dong and Liu 2010). In rice, OsERF3

mediated the resistance to disease and the herbivore-induced defense by modulating multiple hormone signaling pathways (Lu et al. 2011) and OsERF3 could transcriptionally repress the ethylene emission and drought tolerance in rice (Wan et al. 2011), however, its molecular regulatory basis is little known. In this investigation, we found that the expression of *OsERF3* was induced by ABA, ACC, drought and high salinity, which might be associated with the multiple *cis*-elements present in the promoter region of *OsERF3*, providing molecular cues to support its expression characterization in response to different hormones and stresses in rice. We thus have shown that OsERF3 negatively regulated the ethylene emission and drought tolerance in rice through its EAR motif.

OsERF3 belongs to the class II ERF proteins with a conserved EAR repressor motif in the C terminal (Fujimoto et al. 2000). It has been reported that the amino acid residues flanking the EAR motif involved in the regulation of their function. For example, the Ser and Thr residues in many EAR-containing proteins were proved to be phosphorylated in diverse plant species, which might provide the possibility of regulation of EAR motif function by phosphorylation (Hamel et al. 2011). The sequences of the EAR motif are highly conserved among diverse EAR-containing proteins in different plant species, implying this motif play a critical role in regulating their target genes (Kagale and Rozwadowski 2010). Recent studies reported that individual Ala substitution within the EAR motif resulted in partial or complete loss of repression by JAZ8 (Shyu et al. 2012), IAA17 (Tiwari et al. 2004), and chimeric repressors (Hiratsu et al. 2003). The Leu₂₂₇ within the EAR motif of OsERF3 was highly conserved among class II ERFs and Aux/IAA (Tiwari et al. 2004). It has been reported that series stress-related genes (Lu et al. 2011) and ethylene synthesis-related genes were regulated by OsERF3 in rice (Wan et al. 2011).

To investigate whether EAR motif is required for OsERF3 to repress the expression of *OsACS2*, *OsACS6*, and *OsACO2* in rice, we generated transgenic lines over-expressing a mutant version (mEAR) of *OsERF3* in which the Leu₂₂₇ within the EAR motif (DLNRP) was substituted with Ala. Further analyses revealed that the Leu₂₂₇/Ala substitution completely impaired OsERF3 repression activity and subsequently eliminated its repressing expression of *ACO2*, *ACS2*, and *ACS6*, consequently resulting in the increase of ethylene emission and drought tolerance in mEAR lines. For the difference in the repression of *ACS2*, *ACS6*, and *ACO2* in mEAR lines, we speculate it might be related to the complexity of EAR motif. It has been documented that MAPKs could degrade EAR repressors through interacting with the EAR motif and subsequent derepressing target genes in Arabidopsis (Hamel et al. 2011).

The repression of transcription by EAR motif-containing proteins was reported to be governed by epigenetic regulation through direct recruiting corepressors such as TOPLESS (TPL) and AtSAP18, which in turn forms a repression complex with chromatin modifiers (Kagale and Rozwadowski 2011). These results indicate that the EAR motif is an essential repressor domain for OsERF3 to explain its negative regulatory roles in mediating ethylene emission and drought tolerance through affecting the expression of ethylene biosynthesis-related genes in rice. Recently, results have proven that ERF proteins are involved in the ethylene biosynthesis and the ethylene signaling pathway by tuning ethylene synthesis-related genes in different plant species (Zhang et al. 2009; Zhang and Huang 2010; Li et al. 2011). Thus, our data provide new insight into the roles of ERF proteins involved in ethylene biosynthesis and stress responses.

Accession numbers

The GenBank accession numbers are as follows: OsERF3, Os01g0797600; Os05g0497300; Os04g0669200; Os04g0610400; Os06g0691100; Os05g0497200; Os02g0158000; Os02g0189600; AtERF3, AB008105; AtERF4, AB008106; AtERF7, AB032201; AtERF8, AB036884; AtERF9, AB047648; AtERF10, AB047649; AtERF11, AB055882; AtERF12, AB055883.

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