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Transcriptional Regulation of Seven ERFs in Rice Under Oxygen Depletion and Iron Overload Stress

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Abstract Submergence can affect plant development due to the stress of oxygen depletion. Another consequence of submergence is toxicity to plants by an excess soluble iron when the soil is under anaerobic conditions. Currently, much attention has been given to the role of ethylene response transcription factors (ERFs) family, especially when plants respond to conditions of oxygen depletion. Considering such a scenario, we aimed to evaluate the transcriptional response of seven ERF genes in leaves of rice seedlings subjected to conditions of anoxia (N_2), hypoxia (submergence), and iron overload (2,000 mgL^{-1} of $FeSO_4.7H_2O$). All the analyzed genes showed differential transcriptional expression in response to these three stresses. Analyses in the promoter region of these transcription factors indicated that the promoter region closest to the transcription start site is most responsible for the differential response of these genes under conditions of oxygen absence (anoxia) or reduction (hypoxia). On the other hand, the promoter relationship was not detected for iron stress. The methylation of these regions as a mean of regulation is also suggested as well as the possibility of redundancy of different genes expressed at similar times and stressful conditions.

Keywords Epigenetic · Ethylene response factors · *Oryza sativa* · Stress · Transcriptional expression

Abbreviations

AP2/EREBP	APETALA2/ethylene responsive element binding protein
Capes	Coordination for the Improvement of Higher Education Personnel
cDNA	Complementary DNA
CNPq	National Counsel of Technological and Scientific Development
ERFs	Ethylene response factors
$FeSO_4.7H_2O$	Iron sulphate heptahydrate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
MEME	Multiple Motif for In Elicitation
MeV	Multi Experiment Viewer
NCBI BLASTn	The Basic Local Alignment Search Tool from the National Center for Biotechnology Information
N_2	Gaseous nitrogen
Pfam	Protein families
qPCR	Quantitative PCR

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RAP-DB	The Rice Annotation Project Data Base
SK1 and SK2	SNORKEL1 and SNORKEL1
TFBS	Transcription factor binding sites

Introduction

Plants under cultivation are continuously exposed to different stressful conditions. Flooding and submergence are among the abiotic stresses that may adversely affect crop growth, development, and productivity due to conditions of total (anoxia), or partial (hypoxia) lack of O_2, and toxicity due to iron (Fe^{2+}) overload.

Approximately 30 % of the area of Asian rice (*Oryza sativa* L.) and 40 % of African rice are produced in lowland locations where the lack of control over the paddy water level can lead to problems such as temporary submersion of seedlings (Voesenek and Bailey-Serres 2009), a phenomenon known as flash floods (Catling 1992) when water can reach 50 cm in height. Furthermore, Fe^{2+}, which come from a soil rich in this mineral, often increase in soil solution to reach potentially toxic levels during waterlogging (Becana et al. 1998).

It is well known that plant ethylene (C_2H_4) production can be increased by a number of biotic and abiotic stresses (Goeschl et al. 1966; Kawase 1972; Elbeltag and Hall 1974). It is also possible to demonstrate that plant responses to increased ethylene biosynthesis, caused by stress, are related to adaptive responses, helping plant survival under adverse conditions. The ethylene response factors (ERFs), which are primary response elements to ethylene and act as regulators of other specific secondary response genes (Dugardeyn and Van der Straeten 2008), are among the genes most regulated by ethylene. ERFs belong to the AP2/EREBP (APETALA2/ethylene responsive element binding protein) superfamily of transcription factors which are transacting factors at the last step of ethylene signal transduction. The ERFs contain a highly conserved binding domain (Ohme-takagi and Shinshi 1995), which binds only to the target DNA (Allen et al. 1998). Many ERFs have been shown to bind specifically to GCC boxes on gene promoters to modulate the transcription of a wide variety of other ethylene response factors, indicating a transcriptional cascade is involved in ethylene signaling (Ohme-takagi and Shinshi 1995; Buttner and Singh 1997; Zhou et al. 1997; Solano et al. 1998; Gu et al. 2002).

Although rice cannot survive under prolonged submergence, it is one of the few species that can germinate and grow in permanently waterlogged soils (Alpi and Beevers 1983; Perata and Alpi 1993). Therefore, understanding the response of rice to oxygen depletion may suggest mechanisms for increasing tolerance to oxygen depletion in other crops.

Ethylene accumulated in submerged plant tissues triggers a series of response pathways related to stress acclimation (Fukao and Bailey-Serres 2008). In deepwater rice, these routes require regulatory coordination of highly specific ERFs as SNORKEL1 (SK1) and SK2 which then lead to signal transduction routes that regulate responses to anoxia (Jung et al. 2010; Licausi et al. 2010). The *Sub1A-1* allele, which is present in some varieties of rice at the *Sub1* ethylene-response-factor-like locus on chromosome 9 (Fukao et al. 2006; Xu et al. 2006), implicates transcriptional responses of ERF family members in seedlings in conferring submergence tolerance to rice subjected to oxygen depletion (Lasanthi-Kudahettige et al. 2007).

Studies on expression of ERF genes can be employed for understanding plant responses to stresses due to lack of oxygen and Fe^{2+} overload. A study by Nakano et al. (2006) identified 122 putative ERF genes in arabidopsis and 137 in rice. The transcriptional profile for most of these genes is already available in microarray public databases. Although microarrays provide numerous advantages for detection of differentially expressed genes, they have low sensitivity which leads to the need for validation of microarray results through the use of additional techniques such as qPCR (Mei et al. 2003; Lasanthi-Kudahettige et al. 2007).

Heritable physiological changes in metabolic pathways and changes in gene expression are among the sophisticated methods that plants evolved to adapt and survive stress (Boyko and Kovalchuk 2008). Thus, the chromatin state in plants can be modified individually and reversibly by insertion of methyl groups on cytosines trough the action of DNA methyltransferases and also by acetylation, and methylation of the N-terminus of histones to remodel chromatin (Boyko and Kovalchuk 2008; Peng and Zhang 2009). These mechanisms are collectively referred to as epigenetic phenomena. Epigenetic studies are of great interest in plant biology and are expected to become more and more important over time.

The objective of this study is to achieve a more detailed analysis about the transcriptional response of seven ERF genes that had undergone a previous analysis in our laboratory (unpublished data) through Genevestigator meta-analysis (Zimmermann et al. 2008). We also associate such differences in expression profiles with differences in promoter regions of these genes.

Results

Based on qPCR analyses, both types of oxygen depletion stress (anoxia and hypoxia) decreased the level of transcripts of three (*AK062882*, *AK106987*, *AK067195*) of the seven ERF genes (Fig. 1a). The remaining ERF genes behaved differently. Under hypoxia, the ERF gene

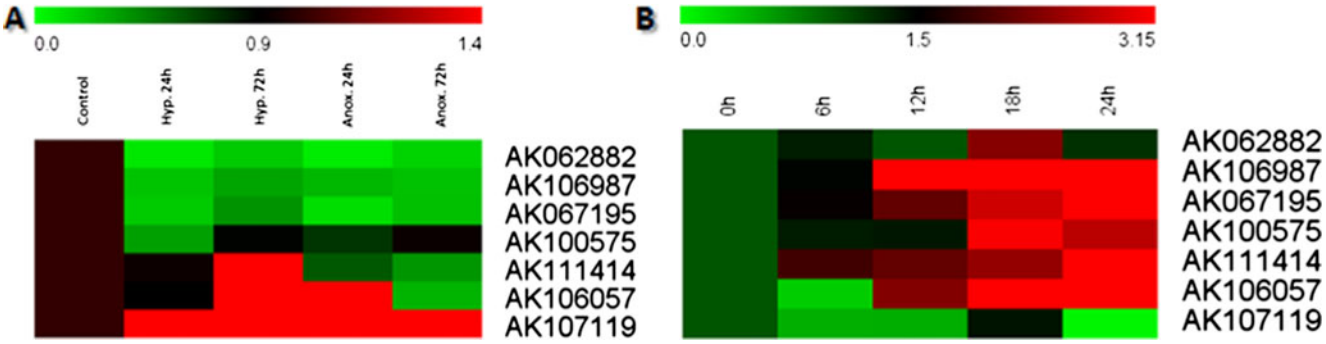

Fig. 1 Transcriptional expression in leaves of rice seedlings. **a** Subjected to oxygen depletion—Heat Map graphic based on relative quantification values from qPCR; **b** Subjected to Fe^{2+} overload—Heat Map graphic based on relative quantification values from qPCR. Expression levels are shown ranging from *green* (*lower*) to *red* (*higher*)

AK100575 exhibited only a transient inhibition for the first 24 h of treatment and then returned by 72 h to normal expression levels (similar to the control). Inhibition of *AK100575* expression was not as strong under anoxia. For ERF *AK111414*, there was a different pattern of differential response to the two oxygen depletion stresses; under hypoxia, *AK111414* showed a slight and transient suppression of transcription at 24 h followed by enhanced transcription at 72 h, whereas under anoxia there was a repression of transcription for this gene. ERF *AK106057* also showed differences between hypoxia and anoxia stresses, with a later induction under hypoxia (72 h). Expression of ERF *AK107119* was strongly induced under both oxygen depletion stresses (Fig. 1a).

Six of the seven rice ERF genes were induced to increased expression under Fe^{2+} overload when compared to the control conditions (Fig. 1b). Only one ERF gene was repressed under this condition. Some ERFs were induced as early as 6 h after plants were treated, but the majority were strongly induced after 12 h. The genes *AK106987*, *AK067195*, *AK100575*, *AK111414* and *AK106057* maintained strong expression levels up to 24 h after the addition of excess iron (stressful condition). The gene *AK062882* was expressed at low levels except for a peak at 18 h after addition of iron excess. Similarly, the gene *AK107119* was inhibited by the addition of iron, but showed an increase at 18 h.

Sequence analysis on the seven ERF genes using MEME (Multiple Motif for In Elicitation), showed multiple conserved motifs in most ERFs at the region between −1,500 and −700 bp (Fig. 2a). However, a displacement of conserved motifs to the proximal region of the promoters was observed when the motifs were compared separately, either within the group of highly repressed genes under oxygen depletion (*AK062882*, *AK106987*, *AK067195*), (Fig. 2b), or within a group of genes which transcriptional expression showed to be variable (*AK100575*, *AK111414*, *AK106057*, *AK107119*) (Fig. 2c).

The amino acid sequences of the studied genes were aligned and the AP2 domains were searched trough Pfam (Punta et al. 2012) and NCBI BLASTn (Altschul et al. 1990). A conserved AP2 domain was found in six of the seven ERFs, but surprisingly, no AP2 domain was found in *AK106987* (Fig. 3). To determine whether similar genes have similar patterns of expression, we aligned the AP2 domains through ClustalW2 to produce a phylogram (Fig. 4a) showing that genes having similar sequences seem to be expressed under similar conditions of oxygen depletion. The amino acid sequence alignments of AP2 domains underlying the phylogram of Fig. 4a grouped genes with similar pattern of expression to anoxic or hypoxic stresses. The phylogram obtained from an analysis of the promoter region from −1,500 to +100 bp (Fig. 4b) did not show gene clusters consistent to expression profiles, which occurred when only the sequences closer to the transcription start site (−500 to +100 bp) were aligned (Fig. 4c). This result is consistent with the previous analysis seen in the heat map and obtained with MEME (Fig. 2).

Comparing the nucleotide alignment from the region between −1,500 and +100 bps of the transcription start site to the expression profiles obtained from the amino acid sequence alignment of these ERF AP2 domains was not consistent indicating a need for further analysis of the promoter region. This led us to an attempt to identify the region of conserved motifs among the seven genes and in two groups with different classes of gene expression. Results obtained from MEME demonstrated conserved CG-rich motifs in the proximal region of promoters, which, considering their importance in the regulation of promoters led us to analyze for the presence of CpG/CpNpG islands (Supplemental Data 1) (Jeddeloh et al. 1998; Vaucheret and Fagard 2001).

Use of PlantPAN revealed CpG/CpNpG islands in the promoters of four ERFs *AK062882*, *AK106987*, *AK067195*, and *AK111414*. Analysis of the CpG/CpNpG islands showed that they occurred in regions similar to the CG-

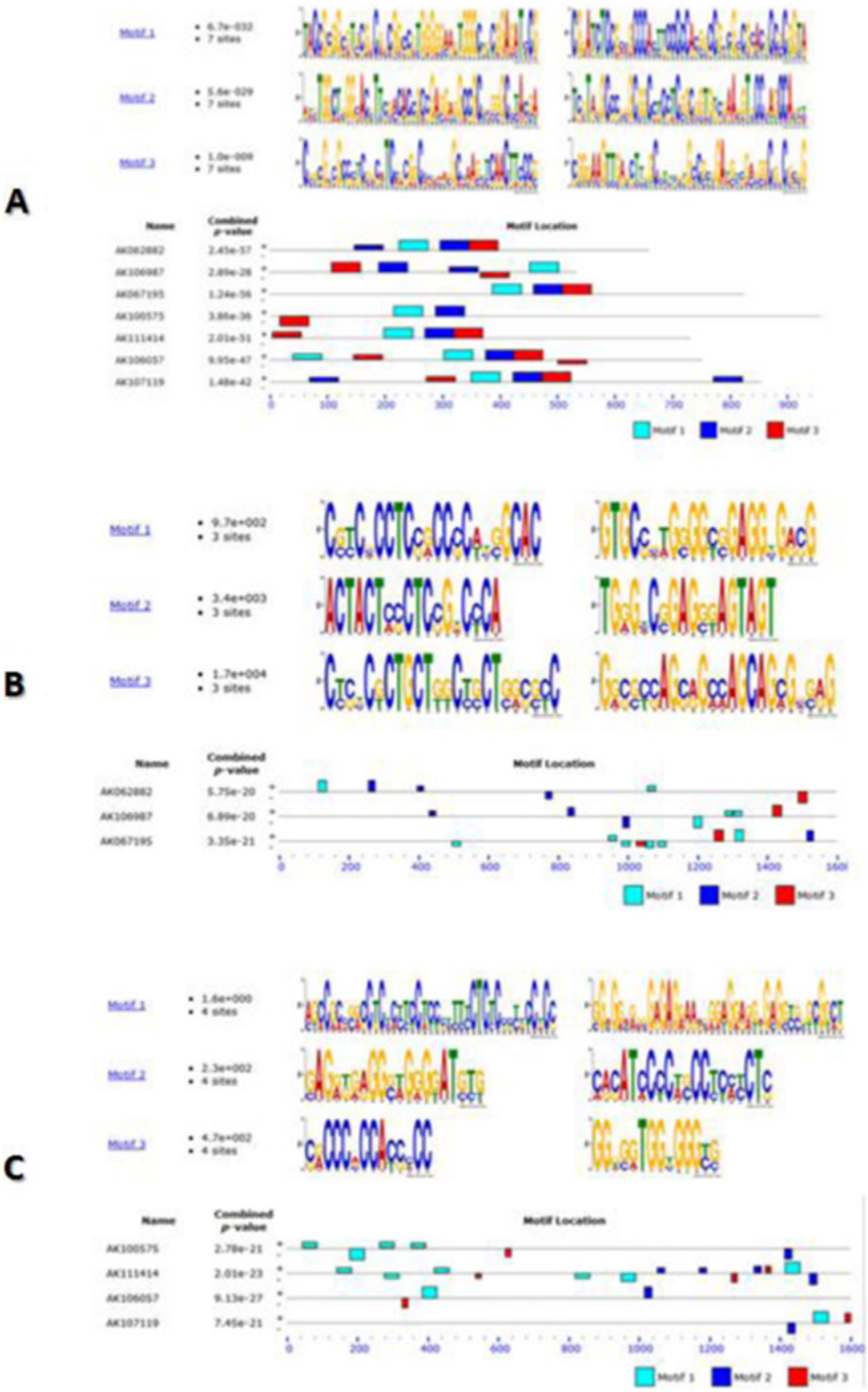

Fig. 2 Conserved motifs analysis at the promoter regions. **a** Region (−1.500 to +100pbs) for the seven studied genes; **b** Region (−1.500 to +100pbs) for the group of three repressed genes under oxygen depletion; **c** Region (−1.500 to +100pbs) for the group of partially or permanently induced under oxygen depletion. *Upward boxes* means that the motif was found in the + strand, *while downward boxes* means that the motif was found in the - strand

rich motifs obtained from MEME promoter analysis for conserved motifs. Three of the four ERFs (*AK062882*, *AK106987*, *AK067195*) displaying CpG/CpNpG islands were all of the genes repressed by oxygen depletion. TFBS present in genes related to acclimation to oxygen depletion were also in these three ERFs (Fig. 5). Collectively, these results suggest that methylation and compression of the proximal promoter region may be responsible for the repression of these genes by preventing access of transcriptional machinery.

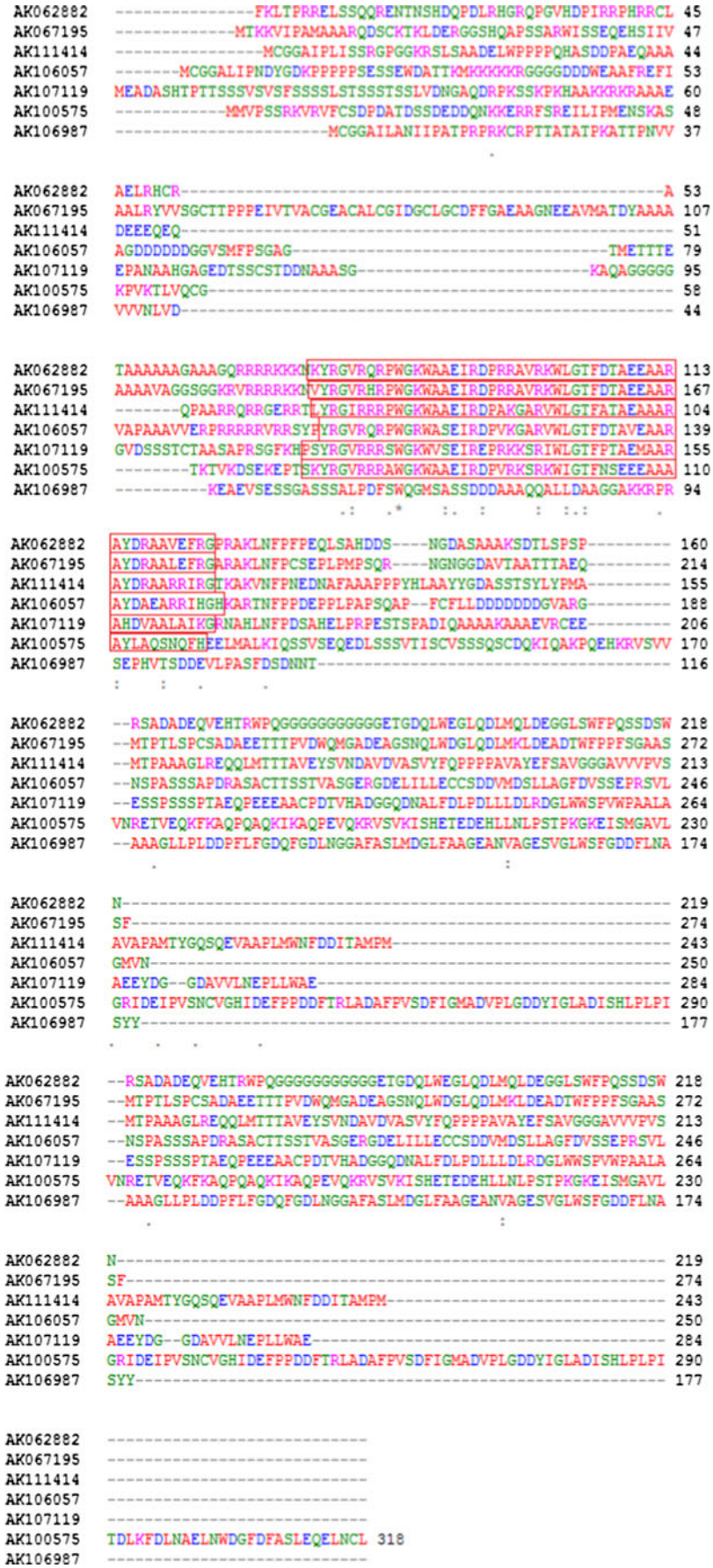

Fig. 3 Protein alignment of the seven ERFs through *ClustalW2* AP2 domains are highlighted inside the *open box*

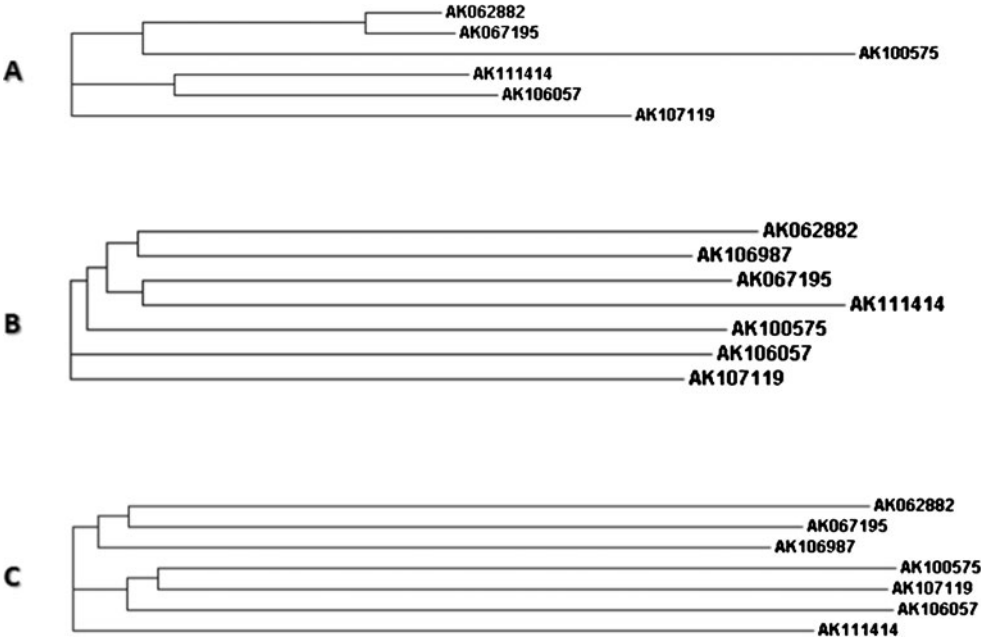

Fig. 4 Phylogram obtained after ClustalW2 analyses **a** Alignment of the six ERF domains; **b** Alignment of the promoter region (−1,500 to +100pbs) nucleotides obtained at TIGR (J. Craig Venter Institute) database; **c** Alignment of the promoter region (−500 to +100pbs) nucleotides obtained at TIGR database

We also used The New Beta PLACE Cis-Element Finder (a program recently developed in our laboratory) in the search for conserved motifs in gene promoters and compared this result with that from PlantPAN. The comparison showed a relatively big difference between the two tools (supplemental materials 3 and 4), nevertheless, it can

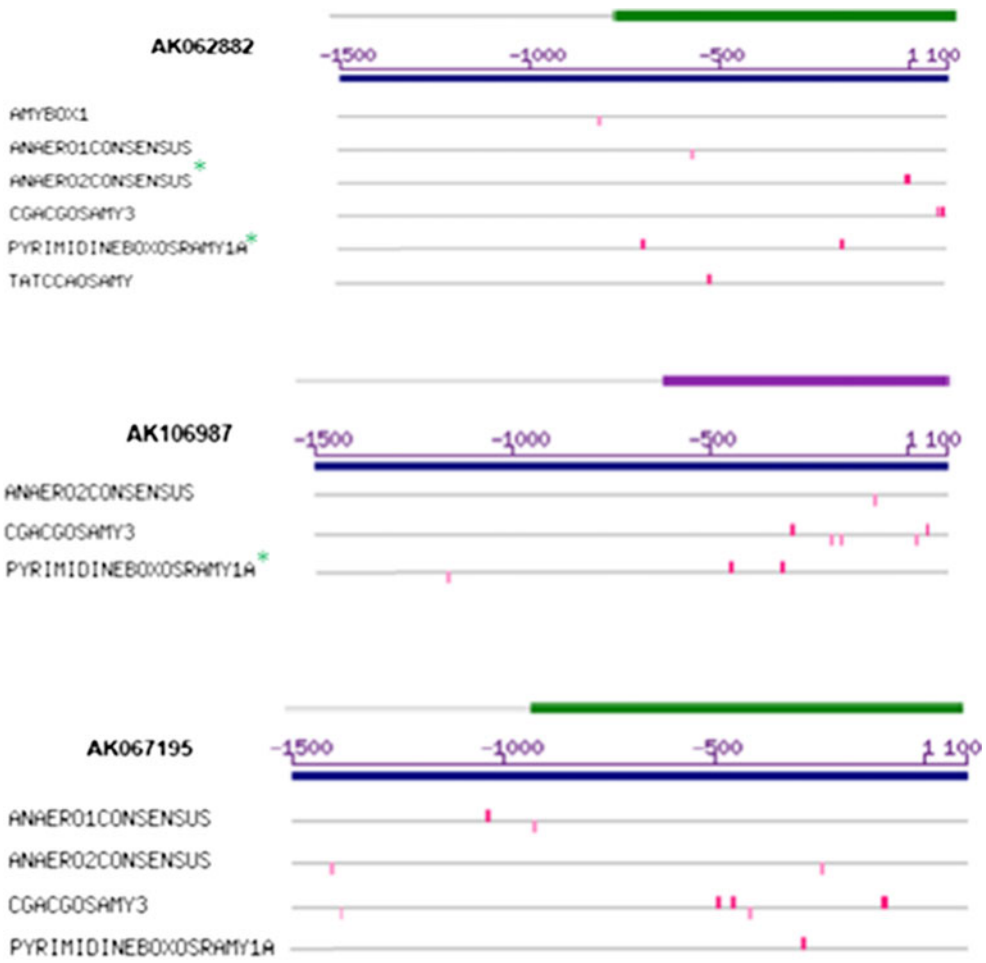

Fig. 5 Transcription factor binding sites associated with anareobiosis found along the promoter region of −1,500 to +100pbs in PlantPAN. TFBS locations are shown as *small pink boxes*. Above each promoter we can se the CpG island also found by PantPAN (*purple boxes* are CpG/CpNpG islands in the sense strand, *green boxes* are CpG/CpNpG islands in the antisense strand). TFBS marked with (*asterisk*) were also found in The New Place

be seen that some motifs related to oxygen depletion were found by both tools (Fig. 5, motifs marked with an asterisk), leading us to believe that these are actually present and probably have an influence on the transcriptional expression of these genes.

Discussion

It has been reported that after rice plant submergence ethylene levels increase rapidly and activate genes associated with stress acclimation responses as well as activating genes associated with carbohydrate metabolism, shoot elongation, and leaf senescence (Fukao et al. 2006; Steffens and Sauter 2005). Therefore, it might be expected that the ERF transcriptional response would greatly differ under conditions of either anoxia or hypoxia since the effects of these stresses on ethylene biosynthesis are completely opposite. In the case of anoxia, ethylene production would cease due the lack of oxygen, which prevents oxidation of ACC (1-aminocyclopropane-1-carboxylic acid). However, it is known that other factors may influence the regulation of ERFs (McGrath et al. 2005; Fukao et al. 2011). This seems to be the case for the genes *AK062882*, *AK106987*, *AK067195*, *AK107195*, *AK107119*, which showed similar regulation in both hypoxia and anoxia. Thus, the action of regulators in addition to ethylene seem to be crucial to oxygen depletion stress.

The activity of genes *AK111414* and *AK106057* may be directly dependent on ethylene since they showed a gradual increase under Fe^{2+} and presented suppression at 72 h of anoxia stress. Under iron excess, the ethylene production is enhanced (Yamauchi and Peng 1995) and under anoxia ethylene cannot be produced (Perata and Voesenek 2007).

In general, higher transcriptional levels were obtained in response to Fe^{2+} overload, reaching a maximum of 3.15 fold while for oxygen depletion the response reached only 1.4 fold (Supplemental material 3). This fact raises the interest in the study of ERF genes in response to iron stress.

The amino acid sequence alignments of AP2 domains (Fig. 4a) that grouped some of the genes with similar pattern of expression considering oxygen depletion stresses appears to be evidence for redundant roles of some ERFs and their simultaneous activation may be required for a dosing effect. This hypothesis has already been raised by Nakano et al. (2006) for ERFs and Birchler and Veitia (2007) for transcription factors in general.

The detection of CpG/CpNpG islands in genes repressed under conditions of low oxygen is an indication that they may be suppressed due to methylation of these regions, with a consequent change in the chromatin state which could prevent transcription factors binding (Stam et al. 1998; Zilberman et al. 2007) followed by transcription.

From the observed results we can say that the present study demonstrates that most of the ERF genes studied respond to stress due to oxygen depletion and Fe^{+2} overload, suggesting an important role of these genes in acclimation responses of rice plants to these stresses. The occurrence of CpG/CpNpG islands near the transcription start site, the same site where conserved motifs between genes of similar expression and TFBS of genes related to anaerobiosis acclimation were found with two different searching tools (New Beta PLACE Cis-Element Finder and PlantPAN) suggest that methylation of these regions may be responsible for transcriptional repression under oxygen depletion conditions.

Investigation of methylation in promoter regions by bisulfite sequencing could shed light on how these genes are regulated. Further investigation through bisulfite sequencing of some promoter regions of these genes should be done, as well as a wider analysis of ERFs transcription trough qPCR to assess if the information about proximal promoter regions shown here is applicable to other members of this transcription factor family. If the proximal promoter elements role in the transcription level of ERFs under oxygen depletion stresses is confirmed a deeper study to identify sequences responsible for this pattern of expression can help us to understand and control gene expression under this stress.

Methods

ERF Identification

Seven ERF genes (Table 1), that according to information available in public databases (*Genevestigator* – http://www.genevestigator.ethz.ch), showed interesting patterns of expression (up or down regulation) under oxygen depletion were selected for further investigation based on qPCR and in silico promoter analysis. The gene *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) was used as a constitutive control for these analyses.

Quantitative PCR (qPCR)

Rice (*Oryza sativa* L. 'Nipponbare') seedlings (15-days-old) were subjected to one of three different treatments for various periods of time from 0 to 72 h: (1) anoxia stress (ultra-pure nitrogen N_2) for 0, 24 and 72 h, (2) hypoxia stress (submergence in ultra-pure water) for 0, 24 and 72 h, or (3) Fe^{2+} overload (2,000 mgL^{-1} of $FeSO_4.7H_2O$) for 0, 6, 12, 18 and 24 h.

Total RNA was extracted from 0.1 g of leaf tissue from rice seedlings following the protocol described by PureLink™ reagent (Invitrogen™, Carlsbad, CA, USA). Samples were treated with DNAse I (Invitrogen™, Carlsbad,

Table 1 Seven ERF genes which showed interesting pattern of expression at *Genevestigator* (http://www.genevestigator.ethz.ch) selected for further analysis trough *qPCR*

TIGR	RAP DB	FL-cDNA	Localization	Generic name
LOC_Os08g36920	*Os08t0474000*	*AK062882*	chr08:23441862..23442942	*OsERF#104#*
LOC_Os03g08490	*Os03t0183200*	*AK106987*	chr03:4366271..4367451	*OsERF#069*
LOC_Os09g28440	*Os09t0457900*	*AK067195*	chr09:18012099..18013198	*OsERF#102*
LOC_Os09g13940	*Os09t0309700*	*AK100575*	chr09:8818014..8821350	*OsERF#109*
LOC_Os03g22170	*Os03t0341000*	*AK111414*	chr03:12761327..12762581	*OsERF#066*
LOC_Os09g11480	*Os09t0287000*	*AK106057*	chr09:7022482..7024039	*OsERF#063*
LOC_Os04g46400	*Os04t0549700*	*AK107119*	chr04:28016357..28017492	*OsERF#033*
LOC_Os04g40950	*Os04t0486600*	*AK064960*	chr04:24804606..24808210	*GAPDH*

TIGR - J. Craig Venter Institute Database; *RAPDB* Rice Annotation Project Database; *FL-cDNA* full length cDNA

CA, USA). The quantity of the RNA was assessed spectroscopically and the quality was assessed through agarose gel electrophoresis. Each sample was reverse-transcribed into cDNA using the commercial kit SuperScript® III First-Strand System for RT-PCR (Invitrogen™, Carlsbad, CA, USA). Samples consisted of cDNA produced from mRNA bulks of the 45 time × treatment seedlings, from which three samples were made as technical replicates. The cDNA quality was assessed trough PCR amplification using oligonucleotides corresponding to *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*).

Primers for the seven ERF genes (Table 2) were designed from sequences deposited in The Rice Annotation Project Data Base (RAP-DB), using Vector NTI Advance™ 11 (Invitrogen™, Carlsbad, CA, USA). The criteria used for primer selection were amplicon size between 50 and 150 bps, CG content between 40 % and 60 %, and melting temperature ranging from 60 to 65 °C according to Applied Biosystems® recommendations.

Dissociation curves were evaluated and only primers giving single peaks and with amplification efficiency close to 100 % were used. qPCR was performed in a 7500 Real-Time PCR System (Applied Biosystems®, Carlsbad, CA, USA) using SYBR® Green (Invitrogen™, Carlsbad, CA, USA) dye. The amplification reaction was carried out in a total volume of 20 μL. Thermal cycle conditions were as follows: 50 °C for 2 min, initial denaturation at 95 °C for 10 min, followed by 40 three-step cycles (95 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min) and final extension at 72 °C for 5 min. Relative quantification of each single gene expression was performed using the comparative threshold cycle method (Livak and Schmittgen 2001). *GAPDH* was used as reference (Lasanthi-Kudahettige et al. 2007) gene to quantify each ERF cDNA abundance. Gene expression data was analyzed trough Multi Experiment Viewer (MeV), EASE Expression Analysis Systematic Explorer version 4.6 (Saeed et al. 2006) and displayed as a heat map graphic (Fig. 1), where 0 h (control) samples were used as calibrators.

Table 2 Primers sequences obtained from Vector NTI Advance™ 11 software, using sequences from RAP-DB

FL-cDNA	Generic name	Primers sequences
AK062882	*OsERF#104*	F: GCCAGCAGCGAGAGAACACAAA
		R: TACTCCTGGTTGCCTCCCATGG
AK106987	*OsERF#069*	F: CGGCGTCATTCGACAGTGACAA
		R: CGAACTGGTCGCCGAACAAGAA
AK067195	*OsERF#102*	F: GCTGTCTCGGGTGCGACTTCTT
		R: AGCAGCATAATCCGTCGCCATT
AK100575	*OsERF#109*	F: CGAGAAGGAACCGACGAGCAAA
		R: GCGGCCTCCTCCTCACTGTTAA
AK111414	*OsERF#066*	F: ACGGCGGTGGAGTACAGCGTTA
		R: ACGGCGCTGAACTCGTAAGCAA
AK106057	*OsERF#063*	F: GTGTGGAGGAGCACTGATCCCG
		R: CGCCACCACGCTTCTTCTTCTT
AK107119	*OsERF#033*	F: CCTCCTCCGTCTCCGTCTCCTT
		R: CGTGTTTGGGCTTGGAGCTCTT
AK064960	*GAPDH*	F: AAGCCAGCATCCTATGATCAGATT
		R: CGTAACCCAGAATACCCTTGAGTTT

F forward; *R* reverse; *RAPDB* Rice Annotation Project Database; *FL-cDNA* full length cDNA

Protein Alignments and Phylogram Design

The similarity between ERF genes were analyzed by aligning their proteins through ClustalW2 using first the entire amino acid sequences and then just their AP2 domains (Larkin et al. 2007). We used default settings to the align amino acid sequences obtained at RAP-DB, changing just the substitution matrix to Dayhoff/PAM for contrasting closely related proteins.

Promoter Alignments and Phylogram Design

The similarity of promoters were analyzed to obtain a phylogram identifying the similarity between these genes by alignment of promoters from −1,500 to +100 bps of these genes, through ClustalW2 (Larkin et al. 2007). Sometimes the analyzed region was changed to −500 to +100 bps, since this region appeared to be the most responsible for differential regulation detected by qPCR in leaves of rice subjected oxygen depletion.

Identification of Conserved Motifs in Promoter Regions

Analysis of the conserved motifs present in promoter regions (−1,500 to +100 bps) of ERF target genes was made trough MEME (http://meme.sdsc.edu/meme4_4_0/intro.html) version 4.4.0 (Bailey et al. 2009).

Analysis of CpG/CpNpG Islands Occurrence

The "Promoter Analysis Tool" from PlantPAN (Chang et al. 2008) was used to assess the CpG/CpNpG island occurrence in promoter regions between −1,500 to +100 bps of each gene.

Identification of Transcription Factor Binding Sites (Cis-Elements)

Transcription factor binding sites (TFBS) in the promoter region of each studied gene was done trough PlantPAN (Chang et al. 2008), which indentified the local of occurrence of each TFBS (Supplemental Data 2), and trough The New PLACE Beta Cis-Element Finder (http://microsatellite.org/cis_input.html), which give us the number of occurrences of each TFBS and its statistical significance (Supplemental Data 3).

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