RESEARCH ARTICLE

Ethylene response factors gene regulation and expression profiles under different stresses in rice

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Received: 24 August 2013; Accepted: 11 December 2013

ABSTRACT: Stresses can cause large yield reductions in cultivated plants. The response to these stresses occurs via a plethora of signalling pathways, where a large number of genes is induced or repressed. Among the environmental stress responsive genes, there are the members of the ethylene response factors (ERF) gene family. The mRNA levels of different ERF are regulated by many hormones and molecules produced under different stress conditions. In this study, with the goal of identifying the response of rice ERF genes to environmental stress, it was analysed the transcriptional expression profile of 114 of these genes under stress by anoxia, salt and *Magnaporthe grisea*. Also, aiming to characterize how the regulation of ERF genes occurs, the amount of known cis regulatory elements in the promoter region of these genes and their association with the expression profiles under the tested conditions were also assessed. The results indicate that some ERF members present the same specific expression profiles under different environmental stresses, while others do not. Within the ERF family, the regulation of gene expression is complex for some genes which have many cis elements in their promoters, but simple for others, demonstrating high levels of divergence among them. The findings demonstrate the importance of the study of each ERF separately, since it is not possible to establish general rules for regulation and probably for the function of these genes.

KEYWORDS: *Oryza sativa*, transcription, cis elements, environmental stresses.

INTRODUCTION

Many biotic and abiotic agents can stress cultivated plants, negatively affecting their yield. However, during evolution, plants developped mechanisms capable of perceiving environmental changes regulating the induction or repression of many genes. The majority of these genes take part in metabolic response pathways, enabling the adaptation to adverse environmental conditions (Yamaguchi-Shinozaki and Shinozaki 2006).

A complex regulatory network is involved in the induction of stress responsive genes (Yamaguchi-Shinozaki and Shinozaki 2006), and a large number of these genes was identified using differential expression analysis in many different species. In the majority of cases, these processes are coordinated by phytohormones, such as ethylene and abscisic acid (Fujimoto et al. 2000).

One of the most important gene families that are related to environmental responses and tolerance is the ethylene response factors (ERF) gene family. Despite its name, these genes are not only regulated by ethylene, but also by other molecules such as abscisic, salicylic, jasmonic, giberellic acids and their interactions (Sakuma et al. 2002, Grennan 2008). ERF genes are defined by the domain AP2/ERF, which consists of aproximately 60 aminoacids involved in DNA binding. The cis element *GCC-Box* (AGCCGCC) is recognized by the majority of ERF members (Ohme-Takagi and Shinshi 1995); however, despite the high conservation on the aminoacid sequence in the ERF binding domain, these factors can also bind other motifs specifically (Nakano et al. 2006, Sasaki et al. 2007, Welsch et al. 2007, Maeo et al. 2009).

Transcription factors belonging to the ERF family have been reported to be involved in many developmental processes (Elliott et al. 1996, Boutilier et al. 2002, Vahala et al. 2013), biotic (Yamamoto et al. 1999) and abiotic (Stockinger et al. 1997, Liu et al. 1999, Xu et al. 2006, Cheng et al. 2013) stress responses (Li et al. 2013). Recently, 122 and 139 ERF genes were identified in arabidopsis and rice, respectively, revealing a strong tendency for redundancy and conservation in this family (Nakano et al. 2006). This tendency for redundancy is still being questioned by studies in which the transcriptional expression of these genes is assessed (Santos et al. 2013).

The ERF family presents well characterized members, with biological and economical importance, such as Dehydration-Responsive Element-Binding proteins (DREBs) (Yamaguchi-Shinozaki and Shinozaki 1994), related to cold, drought and salinity stress. The constitutive expression of *DREB1a* and *DREB1b* in Arabidopsis plants induced the expression of cold-regulated (COR) genes and increased the freezing tolerance (Gilmour et al. 2000, 2004). Similar results were observed for the constitutive action of the protein *DREB2*, under conditions of dehydration and high salinity stress (Liu et al. 1998, Sakuma et al. 2006).

Strong physiological effects are detected not only for DREB genes, but also for other ERF. For example, the superexpression of different ERF genes results in the increased tolerance to hypoxia/anoxia stresses (Xu et al. 2006, Licausi et al. 2010) and drought (Dubouzet et al. 2003). In some rice varieties, the *SUB1A-1* allele induces the negative regulation of ethylene, making plants able to survive complete submergence for prolonged periods (Xu et al. 2006). Besides providing anoxia tolerance, this allele also provides drought and desubmergence (recovering the period after anoxia stress) tolerance. This ability is due to a reduction in water loss by leaves, decrease in lipid peroxidation, gene induction associated with acclimation to dehydration and reduction of reactive oxygen species (ROS) in leaf tissues during drought and desubmergence (Fukao et al. 2011). Similarly, Arabidopsis plants overexpressing the gene *HRE1* (gene belonging to the same group as *SUB1A*) showed increase in anoxia tolerance (Licausi et al. 2010). Although the function of some ERF genes is known, for many of them not even the transcriptional expression is well characterized.

Gene expression is widely controlled in the transcription phase, where the interactions between transcription factors and cis regulatory elements in the promoter region of a given gene perform a crucial role (Brivanlouand Darnell 2002). Therefore, the presence of these elements can determine much of plant stress response, since they act as regulators. The identification of cis elements in the promoter region of ERF genes can auxiliate in the understanding of transcription regulation of these genes under different stress conditions. In this sense, this work aimed to identify cis elements in the promoter regions of ERF genes in rice and to associate the presence of some of these elements to a given expression profile.

MATERIAL AND METHODS

In Silico Expression Profiles of Ethylene Response Factors Genes: ERF members in rice (*Oryza sativa* L.) were identifyied through a previously reported list (Nakano et al. 2006). The *in silico* expression profile of the ERF genes was obtained from public microarray databses using *Genevestigator* (http://www.genevestigator.ethz.ch) (Zimmermann et al. 2008). The evaluated abiotic stresses were: (1) anoxia – Nipponbare plantlet coleoptiles germinated at 28°C, in the dark, with less than 10 μg mL^{-1} of O_2 compared with Nipponbare coleoptiles germinated at 28°C, in the dark (Lasanthi-Kudahettige et al. 2007); and (2) salt – IR29 and FL478 plantlet bases subjected to NaCl and $CaCl_2$ (5:1) for 3 days compared with untreated 30-day-old plants (Walia et al. 2005). Also, the accumulation of ERF gene transcripts under stress caused by rice blast fungus (*Magnaporthe grisea*) was evaluated in Nipponbare at 3 and 4 days after the infection (3 and 4 dpi) (Ribot et al. 2008).

In this study, genes with log2 expression above 0.5 and bellow -0.5 were considered as induced and repressed, respectively. The specificity and overlapping of expression were identified through the use of Venn diagrams (Oliveros 2007).

Identification of Cis Elements in the Promoter Region of Ethylene Response Factors Genes: The sequence corresponding to the promoter region (-1,000 bp before 5'UTR) of each member of the ERF family was obtained from RAP-DB – The Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp/) and was analysed for identification of cis elements.

The identification of cis elements was performed using PLANTCIS software (Maia et al., unpublished data, actually available at http://microsatellite.org/cis_input.html), which searches known cis elements from PLACE - Plant Cis-acting Regulatory DNA Elements (Higo et al. 1999) database. Later, in order to verify if the elements found are not randomly presented, a Z test was performed. In Z test, the difference between occurrence numbers for a given motif (OP) and its average occurrence in the genome is divided by its standard deviation (SD), Z=(OP-OG)/SD. Thus, the probability (p) for each motif was obtained as follows: p=1–z (Table value, at 5% probability). The elements presenting p-values equal to or less than 0.05 were considered as non-random (Rombauts et al. 2003). According to the number of cis elements present in the

ERF promoters, genes were considered as of complex (number of different cis elements above the overall average plus one SD), simple (number of different cis elements bellow the overall average minus one SD) and normal (within the limits established) regulation. Cis elements with higher occurrence among these genes were also reported.

Association Between Gene Expression and Presence of a Given Cis Element: Aiming to verify the association between the presence of a given cis element and a given expression profile, the significant elements were compared with the expression profile obtained for each stress condition. For this comparison, the genes were clustered by their expression profiles using Tocher's method through the generation of a matrix based on Euclydian distances. This analysis was performed using the software GENES (http://www.ufv.br/dbg/genes/gdown1.htm). Later, the presence of common cis elements in the promoter region of genes belonging to the same group was scored.

Analysis of Ethylene Response Factors Gene Expression by Quantitative Polymerase Chain Reaction: For quantitative polymerase chain reaction (qPCR) analysis, three experiments were conducted. Nipponbare rice seedlings (15 day-old) were subjected to different stresses: (1) anoxia for 0, 24, 48 and 72 h; (2) salt (150 mM NaCl) for 0, 8, 16 and 24 h; and (3) *M. grisea* ($1.6x10^5$ spores mL^{-1}) for 0, 3 and 4 dpi.

Tissues from rice seedlings (0.1 g) were collected from the experiments with *M. grisea* (leaves), salt and anoxia (roots). Total RNA was extracted using Trizol® Reagent (Invitrogen™, Carlsbad, CA, USA), according to the protocol described by the manufacturer. To avoid DNA contamination, samples were treated with DNAse I (EC number 3.1.21.1 - Invitrogen™, Carlsbad, CA, USA). The amount and quality of the total RNA were evaluated through spectofotometer and agarose gel electrophoresis. Each sample was reverse transcribed to produce cDNAs using the kit SuperScript® III First-Strand System for RT-PCR (Invitrogen™, Carlsbad, CA, USA), and the quality of cDNA was confirmed by PCR using primers for the Actin gene.

Three ERF genes were randomly selected for the evaluation in qPCR. The primers corresponding to the studied genes (Table 1) were designed from sequences deposited in RAP-DB using the software Vector NTI® Advance 11 (Invitrogen™, Carlsbad, CA, USA). The criteria used for the selection of primers consisted in amplicon size between 50 and 150 bp, GC content between 40 and 60%, and annealing temperature between 60 and 65°C, according to the manufacturer's recommendations (Applied Biosystems®, Carlsbad, CA, USA).

Only primers presenting a dissociation curve with a single peak and amplification efficiency close to 100% were used in this study. Quantitative PCR analysis was performed in a 7500 Real-Time PCR System (Applied Biosystems®, Carlsbad, CA, USA) using SYBR® Green RT-PCR Reagents Kit (Invitrogen™, Carlsbad, CA, USA). The amplification reaction was performed in a total volume of 20 µL. The amplification conditions were: denaturation at 95°C for 10 min, followed by 40 cycles of the routine (95°C for 30 s, 60°C for 1 min and 72°C for 1 min), then by a final extension at 72°C for 5 min. The relative expression of each gene was obtained according to the previously described method (Pfaffl 2001). For each analysed gene, a normalizing procedure was used having the *Actin* gene and the zero time as controls.

RESULTS

Identification of Ethylene Response Factors Genes: The ERF genes analysed in this work were initially described by Nakano et al. (2006), who identified 139 genes in rice from TIGR (MSU Rice Genome Annotation Project Team) database. From the 139 identified genes, 114 presented expression profile in Genevestigator (Figure 1, Table 2). Genes were divided into two groups (54 and 60 genes each) to facilitate the presentation of the results.

Expression Profile of Ethylene Response Factors Genes under Stress Conditions: The expression profiles of rice ERF genes (Figure 1) indicated that a portion of these genes respond to *M. grisea* stress three days after infection (3 dpi). In this condition, 28 genes were induced, 2 genes were repressed, and no change was detected for the other 84 genes (Table 3).

Table 1. Primers used for quantitative polymerase chain reaction analyses

Primers	Forward	Reverse
Os02g43790	5'TCACGCGCGCTCCTCAACTT3'	5'AGAAGAGCCGGAGCTCGCC3'
Os01g21120	5'AGGAGCTGCTCGCGTACGAGAA3'	5'AGCGACGGCAGCTCGTAGTCTT3'
Os09g11480	5'CATCCACGGCCACAAGGCAA3'	5'TCGTCGAGCAGGAAGCAGAACG3'
Actin	5'CAGCCACACTGTCCCCATCTA3'	5'AGCAAGGTCGAGACGAAGGA3'

Regarding the second infection time (4 dpi), 42 genes were induced and 6 were repressed while the other 66 genes did not change its expression levels. Anoxia stress caused the induction of 22 genes and the repression of 31 genes while in the remaining genes no changes were observed. Salt stress showed the smallest effect on the regulation of the analysed genes. No induced gene was detected, only eight were repressed.

Venn diagrams (Figure 2) demonstrate overlapping regulation of ERF genes responsive to the studied stresses. Comparing genes induced by *M. grisea* at different infection times, an overlap in expression of 26 genes was found (Table 4). This result was expected, since the stress is the same, only altering the exposition time. Anoxia, *M. grisea* 3 dpi and *M. grisea* 4 dpi caused common induction of three genes, which are related with patogenicity, revealing an overlapping expression between biotic and abiotic stresses. The anoxia and *M. grisea* 3 dpi caused the induction of four common genes while anoxia and *M. grisea* 4 dpi caused the common induction of ten genes (Figure 2A).

Unique expression profiles were also observed in each stress (Table 5). A high number of uniquely induced genes was found under anoxia (11). Genes induced only by *M. grisea* at 3 dpi (1) and genes induced only by *M. grisea* at 4 dpi (9) were also found.

For the repressed genes, it was observed that under salt and anoxia stresses, there was a common repression for five genes (Table 4). When the anoxia and *M. grisea* 3 dpi stresses were applied, the repression of one common gene was observed and when the stresses by anoxia and *M. grisea* 4 dpi were compared, two genes were commonly repressed. When the stresses by anoxia, *M. grisea* 3 dpi and *M. grisea* 4 dpi were compared, one gene was commonly regulated (Figure 2B). Only one gene was commonly repressed under the stresses of *M. grisea* 3 dpi and *M. grisea* 4 dpi. It is proposed that genes commonly repressed under different stresses conditions are more likely to play a general role in adaptation to stresses.

A specific repression profile is observed for ERF, according to the studied stress. The major group of uniquely repressed genes (24) was found under anoxia conditions. On the other hand, just three genes were repressed only under salt stress. For *M. grisea* infection, one gene was uniquely repressed at 3 dpi and four at 4 dpi.

From the expression data, the ERF genes were clustered according to their similarity in response to each stress condition. Under anoxia, salt and *M. grisea*, the genes were divided into eigth, three and six groups, respectively (Table 6).

Identification of Cis Elements in Promoter Regions of Ethylene Response Factors Genes: From 139 ERF genes predicted by Nakano et al. (2006), 115 were used for obtaining promoters and identifying cis elements. The remaining genes (24) presented annotation problems or were not identified in RAP-DB (Table 2).

In the 115 analysed promoters, a total of 250 different significant cis elements ($p \leq 5\%$) were found, with an occurrence of at least one per gene. An average of 14 and a SD of 5 different cis elements were found. In order to establish

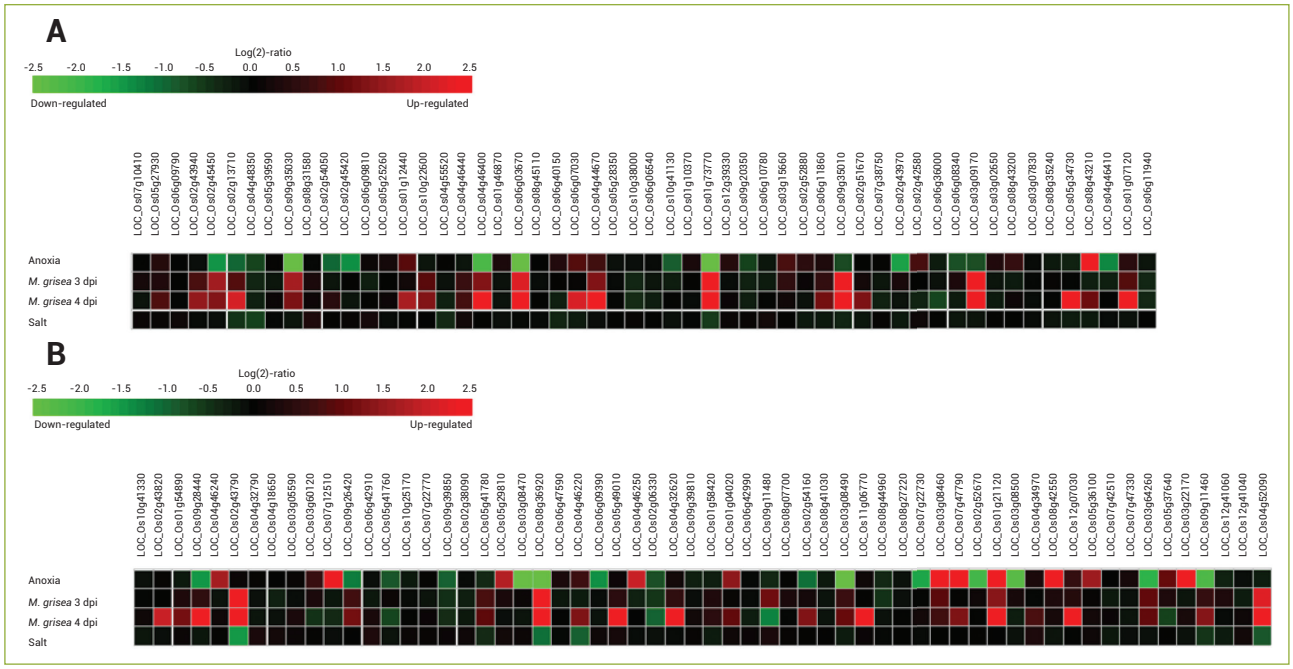

Figure 1. Expression profile of the ethylene response factors gene family members in rice seedlings subjected to different stresses. The expression profile is presented as a heat map, generated by Genevestigator. (A) group 1; (B) group 2.

a pattern of regulation, genes with a number of different cis elements higher than 19, bellow 9 and between 9 and 19 were considered of complex, simple and normal regulation, respectively. According to Table 7, 18 and 13 genes were found to be of complex and simple regulation, respectively. It is suggested that for genes of simple regulation, a small interaction of transcription factors is needed, while for the activation of genes with complex regulation, a higher interaction between these factors is probably required.

In this work, cis elements with frequencies ranging from 1 to 159 were found in the ERF family. The elements that occurred 20 times or more within the whole ERF family (sum of all occurrences from all analysed genes) are shown in Table 8.

Association of Cis Elements with the Expression Profile of Ethylene Response Factors Genes under Different Stress Conditions: No common cis elements were found in the promoter regions when compared: (1) the four genes commonly induced by anoxia and *M. grisea* 3 dpi; (2) the 10 genes commonly induced by anoxia and *M. grisea* 4 dpi; (3) the 26 genes commonly induced by *M. grisea* 3 and 4 dpi (Figure 2A); (4) the one commonly repressed gene under anoxia and *M. grisea* 3 dpi; (5) the one commonly repressed gene under anoxia and *M. grisea* 4 dpi; (6) the five genes repressed under salt and anoxia stresses (Figure 2B).

The analysis of clusters formed by expression similarity within each stress (Table 6) indicated that, under anoxia stress,

Table 2. Ethylene response factors genes identified in rice

Genes MSU TIGR				
Os04g44670	*Os04g48330*[a]	*Os06g08340*	*Os05g41780*	*Os02g43820*
Os02g42580	*Os03g02650*	*Os02g10760*[a]	*Os01g58420*	*Os10g41330*[f]
Os03g09170	*Os10g38000*	*Os02g55380*[a]	*Os04g57340*[a]	*Os04g46240*[e]
Os08g31580	*Os09g35030*	*Os04g56150*[a]	*Os04g52090*	*Os02g34260*[a]
Os02g51670	*Os02g45450*	*Os12g39330*	*Os06g47590*	*Os01g64790*[a]
Os09g20350[c]	*Os06g03670*	*Os07g10410*	*Os05g41760*	*Os04g34970*
Os06g11860	*Os01g73770*	*Os07g38750*	*Os02g06330*[d]	*Os04g32620*
Os10g22600	*Os08g43200*[b]	*Os01g12440*	*Os02g09650*[af]	*Os11g06770*
Os05g49700[a]	*Os08g43210*[b]	*Os01g46870*	*Os07g47330*	*Os12g07030*
Os06g07030	*Os04g48350*	*Os06g06540*[e]	*Os02g38090*	*Os02g34270*[ad]
Os04g55520	*Os09g35010*[f]	*Os05g25260*	*Os08g07700*	*Os09g28440*
Os03g15660	*Os09g35020*[a]	*Os07g12510*	*Os02g32040*[a]	*Os02g52670*
Os06g09690[ad]	*Os02g43940*	*Os03g60120*	*Os04g32790*	*Os08g36920*
Os02g54050	*Os04g46400*	*Os10g25170*	*Os03g64260*	*Os05g36100*[c]
Os08g35240[a]	*Os04g46440*	*Os03g08460*	*Os05g49010*	*Os08g42550*
Os06g11940[e]	*Os02g43970*	*Os05g29810*	*Os05g37640*[c]	*Os02g32140*[a]
Os06g09810[d]	*Os10g41130*	*Os03g08470*	*Os07g22770*	*Os01g04020*
Os06g09790[d]	*Os04g46410*	*Os09g11480*	*Os09g39850*	*Os09g13940*[a]
Os06g09760[ad]	*Os06g36000*	*Os03g08500*	*Os03g05590*	*Os08g27220*[b]
Os06g10780[d]	*Os02g13710*	*Os07g42510*	*Os10g30840*[ad]	*Os12g41030*[ad]
Os02g52880	*Os01g10370*	*Os03g22170*	*Os08g44960*[d]	*Os12g41040*[d]
Os06g09730[ad]	*Os01g07120*	*Os07g47790*	*Os09g39810*	*Os12g41060*
Os01g66270[a]	*Os03g07830*	*Os01g21120*	*Os04g18650*	*Os06g42910*
Os05g34730	*Os05g27930*	*Os03g08490*	*Os07g22730*	*Os06g42990*[e]
Os11g13840[a]	*Os05g39590*	*Os02g54160*	*Os02g43790*	*Os08g41030*
Os02g35240	*Os08g45110*	*Os06g09390*	*Os01g54890*	-[ad]
Os04g36640[a]	*Os05g28350*	*Os09g26420*	*Os04g46220*	-[ad]
Os02g45420	*Os06g40150*	*Os09g11460*	*Os04g46250*	

[a]Do not present information in Genevestigator; [b]Different genes in MSU TIGR correspond to the same gene in RAP-DB; [c]Different genes in RAP-DB correspond to the same gene in MSU TIGR; [d]No corresponding gene in RAP-DB; [e]No description and/or assembly error in RAP-DB; [f]No protein sequence in RAP-DB.

Table 3. Description of the results obtained from the digital expression profile of rice ethylene response factors genes*

Induced in *M. grisea* infection (3 dpi)	Repressed in *M. grisea* infection (3 dpi)	Induced in *M. grisea* infection (4 dpi)	Repressed in *M. grisea* infection (4 dpi)	Induced in anoxia stress	Repressed in anoxia stress	Repressed in salt
LOC_Os02g43790	*LOC_Os08g44960*	*LOC_Os02g43790*	*LOC_Os03g60120*	*LOC_Os03g08460*	*LOC_Os03g08490*	*LOC_Os02g13710*
LOC_Os01g73770	*LOC_Os02g06330*	*LOC_Os03g09170*	*LOC_Os05g37640*	*LOC_Os08g43210*	*LOC_Os08g36920*	*LOC_Os09g11460*
LOC_Os08g36920		*LOC_Os09g35010*	*LOC_Os06g36000*	*LOC_Os07g47790*	*LOC_Os01g73770*	*LOC_Os01g73770*
LOC_Os09g35010		*LOC_Os09g28440*	*LOC_Os05g41760*	*LOC_Os03g22170*	*LOC_Os03g08470*	*LOC_Os04g48350*
LOC_Os03g09170		*LOC_Os08g36920*	*LOC_Os02g06330*	*LOC_Os01g21120*	*LOC_Os09g35030*	*LOC_Os04g52090*
LOC_Os04g52090		*LOC_Os04g52090*	*LOC_Os09g11480*	*LOC_Os08g42550*	*LOC_Os06g03670*	*LOC_Os04g46220*
LOC_Os06g03670		*LOC_Os06g03670*		*LOC_Os07g12510*	*LOC_Os03g08500*	*LOC_Os08g36920*
LOC_Os09g35030		*LOC_Os01g73770*		*LOC_Os04g46250*	*LOC_Os09g11460*	*LOC_Os02g43790*
LOC_Os02g45450		*LOC_Os04g32620*		*LOC_Os05g29810*	*LOC_Os04g46400*	
LOC_Os01g21120		*LOC_Os05g34730*		*LOC_Os04g46240*	*LOC_Os02g52670*	
LOC_Os04g46400		*LOC_Os01g21120*		*LOC_Os05g36100*	*LOC_Os03g64260,*	
LOC_Os04g44670		*LOC_Os12g07030*		*LOC_Os01g04020*	*LOC_Os07g22730*	
LOC_Os09g11480		*LOC_Os01g07120*		*LOC_Os05g37640*	*LOC_Os02g43970*	
LOC_Os03g64260		*LOC_Os11g06770*		*LOC_Os01g12440*	*LOC_Os09g28440*	
LOC_Os03g08460		*LOC_Os05g49010*		*LOC_Os06g07030*	*LOC_Os02g45450*	
LOC_Os10g22600		*LOC_Os04g44670*		*LOC_Os03g15660*	*LOC_Os06g09390*	
LOC_Os01g07120		*LOC_Os04g46400*		*LOC_Os02g42580*	*LOC_Os02g45420*	
LOC_Os02g13710		*LOC_Os06g07030*		*LOC_Os04g44670*	*LOC_Os04g46410*	
LOC_Os05g41780		*LOC_Os02g13710*		*LOC_Os04g46220*	*LOC_Os09g26420*	
LOC_Os02g43940		*LOC_Os02g43820*		*LOC_Os03g60120*	*LOC_Os02g54160*	
LOC_Os09g26420		*LOC_Os01g12440*		*LOC_Os12g07030*	*LOC_Os02g54050*	
LOC_Os03g08490		*LOC_Os02g43940*		*LOC_Os08g43200*	*LOC_Os02g13710*	
LOC_Os04g46440		*LOC_Os09g11460*			*LOC_Os09g39850*	
LOC_Os09g11460		*LOC_Os02g45450*			*LOC_Os10g41130*	
LOC_Os06g11860		*LOC_Os10g22600*			*LOC_Os05g41760*	
LOC_Os09g28440		*LOC_Os02g54160*			*LOC_Os06g08340*	
LOC_Os03g15660		*LOC_Os07g47790*			*LOC_Os02g06330*	
LOC_Os04g32620		*LOC_Os04g46220*			*LOC_Os03g09170*	
		LOC_Os02g51670			*LOC_Os09g35010*	
		LOC_Os09g35030			*LOC_Os04g48350*	
		LOC_Os01g54890			*LOC_Os09g20350*	
		LOC_Os06g11860				
		LOC_Os09g26420				
		LOC_Os08g43210				
		LOC_Os03g08490				
		LOC_Os03g64260				
		LOC_Os01g04020				
		LOC_Os05g41780				
		LOC_Os05g27930				
		LOC_Os04g34970				
		LOC_Os04g46440				
		LOC_Os03g08460				

*Salt stress showed the smallest effect on the regulation of the analysed genes. No induced gene was detected.

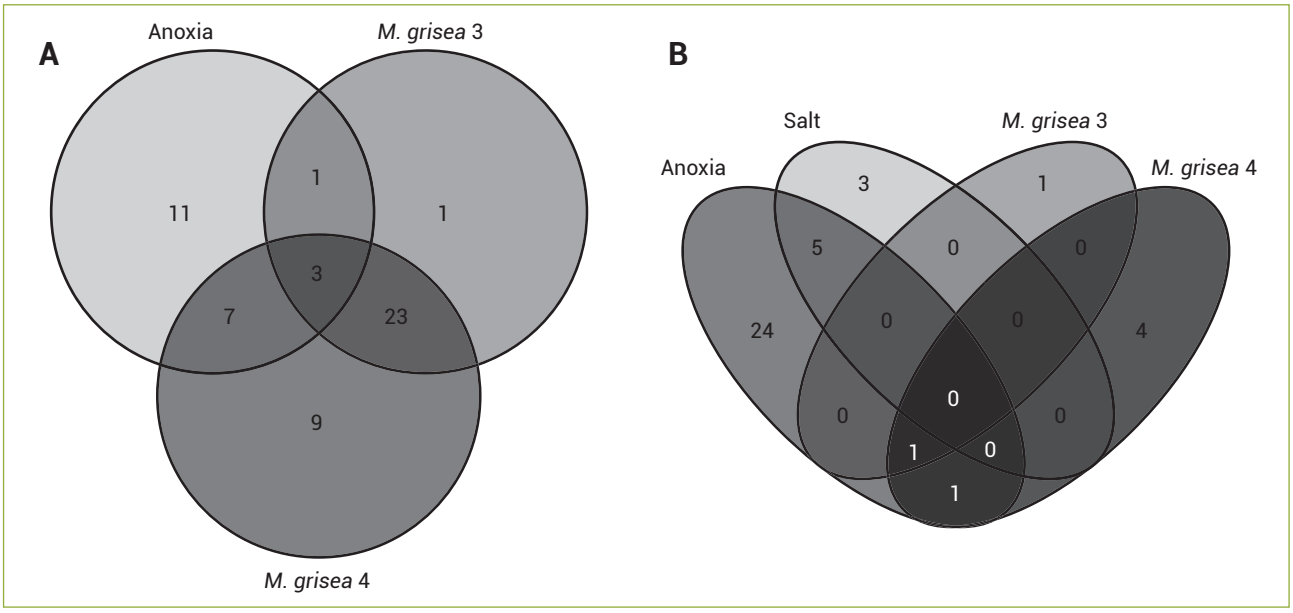

Figure 2. Specificity for induced (A) and repressed (B) genes in rice.

Table 4. Overlapping regulation under different stresses obtained from the digital expression profile of rice ethylene response factors genes

Overlapping regulation in *M. grisea* at different infection times	Overlapping induction in anoxia, *M. grisea* 3 dpi and *M. grisea* 4 dpi	Overlapping induction in anoxia and *M. grisea* 3 dpi	Overlapping induction in anoxia and *M. grisea* 4 dpi	Overlapping repression in salt and anoxia	Overlapping repression in anoxia and *M. grisea 3* dpi	Overlapping repression in anoxia and *M. grisea* 4 dpi	Overlapping repression in anoxia, *M. grisea* 3 dpi and *M. grisea* 4 dpi	Overlapping repression in *M. grisea* 3 dpi and *M. grisea* 4 dpi
LOC_Os04g32620	*LOC_Os01g21120*	*LOC_Os03g15660*	*LOC_Os12g07030*	*LOC_Os08g36920*	*LOC_Os02g06330*	*LOC_Os02g06330*	*LOC_Os02g06330*	*LOC_Os02g06330*
LOC_Os09g28440	*LOC_Os04g44670*	*LOC_Os04g44670*	*LOC_Os06g07030*	*LOC_Os01g73770*		*LOC_Os05g41760*		
LOC_Os06g11860	*LOC_Os03g08460*	*LOC_Os01g21120*	*LOC_Os01g12440*	*LOC_Os09g11460*				
LOC_Os09g11460		*LOC_Os03g08460*	*LOC_Os07g47790*	*LOC_Os02g13710*				
LOC_Os04g46440			*LOC_Os04g46220*	*LOC_Os04g48350*				
LOC_Os03g08490			*LOC_Os08g43210*					
LOC_Os09g26420			*LOC_Os01g04020*					
LOC_Os02g43940			*LOC_Os01g21120*					
LOC_Os05g41780			*LOC_Os04g44670*					
LOC_Os02g13710			*LOC_Os03g08460*					
LOC_Os01g07120								
LOC_Os10g22600								
LOC_Os03g64260								
LOC_Os04g46400								
LOC_Os02g45450								
LOC_Os09g35030								
LOC_Os06g03670								
LOC_Os04g52090								
LOC_Os03g09170								
LOC_Os09g35010								
LOC_Os08g36920								
LOC_Os01g73770								
LOC_Os02g43790								
LOC_Os04g44670								
LOC_Os01g21120								
LOC_Os03g08460								

the promoters of genes belonging to group three showed four cis elements in common (Table 9):

REALPHALGLHCB21:AACCAA;
HEXMOTIFTAH3H4:ACGTCA;
TATAPVTRNALEU:TTTATATA
ACGTABOX:TACGTA.

These elements also appear in other promoters, but not in the same combination, suggesting that the regulation observed for genes *LOC_Os03g08470* and *LOC_Os09g35030* under anoxia may be performed by these four elements together.

Also under anoxia, promoters of genes belonging to group five presented one common cis element, CACGTGMOTIF:CACGTG. This might be an indication that the expression profile of *LOC_Os08g36920* and *LOC_Os01g73770* observed under this condition is dependent on the combination of other elements present in these promoters.

Under salt stress, three clusters of ERF genes were formed, but no cis elements common to genes within groups were found (Table 9).

Under *M. Grisea* stress, it was detected two common cis elements (TATAPVTRNALEU:TTTATATA and RBCSCONSENSUS:AATCCAA) among the genes belonging to group 2 (Table 9). These results suggest that the interaction between transcription factors and these two elements contributes for the regulation observed in *LOC_Os02g45450* and *LOC_Os09g35030* under these conditions.

Ethylene Response Factors Gene Expression in Rice Detected by Quantitative Polymerase Chain Reaction: In this work, the quantitative expression of three out of 139 ERF genes was evaluated under three stresses (salt, anoxia and *M. grisea*) (Figure 3). The gene *LOC_Os09g11480* (*EREBP 5*) exibited an increase in transcript levels when exposed to salt, except at 16 h, when

Table 5. Uniquely regulated under a certain stress. Data obtained from the digital expression profile of rice ethylene response factors genes

Induced under anoxia	Induced under *M. grisea* at 3 dpi	Induced under *M. grisea* at 4 dpi	Repressed under anoxia	Repressed under salt	Repressed under *M. grisea* at 3 dpi	Repressed under *M. grisea* at 4 dpi
LOC_Os08g43200	*LOC_Os09g11480*	*LOC_Os04g34970*	*LOC_Os03g08490*	*LOC_Os02g43790*	*LOC_Os08g44960*	*LOC_Os09g11480*
LOC_Os03g60120		*LOC_Os05g27930*	*LOC_Os03g08470*	*LOC_Os04g46220*		*LOC_Os06g36000*
LOC_Os02g42580		*LOC_Os01g54890*	*LOC_Os09g35030*	*LOC_Os04g52090*		*LOC_Os05g37640*
LOC_Os05g37640		*LOC_Os02g51670*	*LOC_Os06g03670*			*LOC_Os03g60120*
LOC_Os05g36100		*LOC_Os02g54160*	*LOC_Os03g08500*			
LOC_Os04g46240		*LOC_Os02g43820*	*LOC_Os04g46400*			
LOC_Os05g29810		*LOC_Os05g49010*	*LOC_Os02g52670*			
LOC_Os04g46250		*LOC_Os11g06770*	*LOC_Os03g64260*			
LOC_Os07g12510		*LOC_Os05g34730*	*LOC_Os07g22730*			
LOC_Os08g42550			*LOC_Os02g43970*			
LOC_Os03g22170			*LOC_Os09g28440*			
			LOC_Os02g45450			
			LOC_Os06g09390			
			LOC_Os02g45420			
			LOC_Os04g46410			
			LOC_Os09g26420			
			LOC_Os02g54160			
			LOC_Os02g54050			
			LOC_Os09g39850			
			LOC_Os10g41130			
			LOC_Os06g08340			
			LOC_Os03g09170			
			LOC_Os09g35010			
			LOC_Os09g20350			

Table 6. Gene clusters formed according to the expression profiles of ethylene response factors under several stress conditions

Stress	Group	Genes
Anoxia	1	*LOC_Os06g09390, LOC_Os02g45420, LOC_Os02g45450, LOC_Os09g28440, LOC_Os04g46410, LOC_Os02g43970, LOC_Os07g22730, LOC_Os09g26420, LOC_Os02g54160, LOC_Os03g64260, LOC_Os02g52670, LOC_Os04g46400, LOC_Os09g11460, LOC_Os03g08500, LOC_Os06g03670*
	2	*LOC_Os04g46240, LOC_Os05g36100, LOC_Os01g04020, LOC_Os05g29810, LOC_Os04g46250, LOC_Os05g37640, LOC_Os07g12510, LOC_Os08g42550*
	3	*LOC_Os03g08470, LOC_Os09g35030*
	4	*LOC_Os01g21120, LOC_Os03g22170, LOC_Os07g47790, LOC_Os08g43210*
	5	*LOC_Os08g36920, LOC_Os01g73770*
	6	*LOC_Os03g08490*
	7	*LOC_Os03g08460*
	8	Remaining genes
Salt	1	*LOC_Os02g13710, LOC_Os09g11460, LOC_Os01g73770, LOC_Os04g48350, LOC_Os04g52090, LOC_Os04g46220*
	2	*LOC_Os02g43790, LOC_Os08g36920*
	3	Remaining genes
M. grisea	1	*LOC_Os04g46400, LOC_Os04g44670, LOC_Os01g21120, LOC_Os01g07120, LOC_Os02g13710, LOC_Os05g49010, LOC_Os12g07030, LOC_Os04g32620, LOC_Os09g28440, LOC_Os11g06770, LOC_Os05g34730*
	2	*LOC_Os02g45450, LOC_Os09g35030*
	3	*LOC_Os06g03670, LOC_Os04g52090, LOC_Os09g35010, LOC_Os03g09170, LOC_Os08g36920, LOC_Os01g73770*
	4	*LOC_Os02g43790*
	5	*LOC_Os09g11480*
	6	Remaining genes

Table 7. Number of different cis elements in promoter regions of genes considered with complex and simple regulation

Complex regulation	Cis amounts	Simple regulation	Cis amounts
LOC_Os04g34970	20	*LOC_Os02g42580*	3
LOC_Os05g49700	20	*LOC_Os01g46870*	6
LOC_Os06g07030	20	*LOC_Os02g51670*	6
LOC_Os10g25170	21	*LOC_Os03g15660*	6
LOC_Os01g21120	22	*LOC_Os02g52880*	7
LOC_Os05g27930	22	*LOC_Os05g28350*	7
LOC_Os07g47790	22	*LOC_Os10g41330*	7
LOC_Os08g45110	22	*LOC_Os02g34260*	8
LOC_Os10g22600	22	*LOC_Os03g02650*	8
LOC_Os08g07700	24	*LOC_Os04g32620*	8
LOC_Os12g07030	24	*LOC_Os04g36640*	8
LOC_Os02g43790	25	*LOC_Os04g48330*	8
LOC_Os02g10760	26	*LOC_Os09g13940*	8
LOC_Os08g31580	26		
LOC_Os03g08470	29		
LOC_Os08g36920	30		
LOC_Os08g41030	30		
LOC_Os06g03670	31		

Table 8. Sum of occurrences of each cis element in all analysed genes (only genes with 20 or more occurrences are shown)

Cis element	Occurrences	Cis element	Occurrences
ABREOSRAB21	20	MYBPZM	29
ARR1AT	20	IBOXCORE	30
INRNTPSADB	20	RAV1AAT	30
MYCATRD22	20	WBOXATNPR1	30
NAPINMOTIFBN	20	WRKY71OS	30
RYREPEATVFLEB4	20	RYREPEATGMGY2	33
TATABOX3	20	REALPHALGLHCB21	34
ARFAT	21	PRECONSCRHSP70A	36
POLLEN1LELAT52	21	RYREPEATLEGUMINBOX	36
WBOXHVISO1	21	ACGTABOX	38
-300ELEMENT	22	SORLIP1AT	41
BOXCPSAS1	22	GTGANTG10	44
CARGCW8GAT	22	POLASIG1	44
GT1CORE	22	BIHD1OS	47
NODCON1GM	22	RYREPEATBNNAPA	47
OSE1ROOTNODULE	22	CCAATBOX1	49
SEF4MOTIFGM7S	22	ABRERATCAL	50
EECCRCAH1	23	CURECORECR	52
HEXAMERATH4	23	GCCCORE	52
RBCSCONSENSUS	23	CGACGOSAMY3	54
WBOXNTERF3	23	RHERPATEXPA7	54
CACGTGMOTIF	24	EBOXBNNAPA	60
ABRELATERD1	25	MYCCONSENSUSAT	60
ACGTABREMOTIFA2OSEM	25	GATABOX	66
DPBFCOREDCDC3	25	GT1CONSENSUS	67
HEXMOTIFTAH3H4	26	CAATBOX1	70
ROOTMOTIFTAPOX1	27	LTRECOREATCOR15	81
TAAAGSTKST1	27	DOFCOREZM	85
SORLIP2AT	28	ACGTATERD1	108
GT1GMSCAM4	29	CACTFTPPCA1	127
MYB1AT	29	CGCGBOXAT	159

Table 9. Common cis elements among the gene groups formed according to the expression profile

Stress	Group	Common cis elements
Anoxia	3	REALPHALGLHCB21; HEXMOTIFTAH3H4; TATAPVTRNALEU; ACGTABOX
	5	CACGTGMOTIF
Salt	-	-
M. grisea	2	TATAPVTRNALEU; RBCSCONSENSUS

a decrease was observed. *LOC_Os01g21120* (*EREBP 3*) and *LOC_Os02g43790* (*ERF 91*) presented a decrease in the expression along the time of exposure to salt stress conditions, but at 24 h a slight increase was observed.

Under anoxia stress, the gene *LOC_Os09g11480* presented a linear increase in transcript levels along the time. It is interesting to note that this gene presented an increase in transcript levels close to 600-fold. The genes *LOC_Os01g21120* and *LOC_Os02g43790* presented a similar performance under lack of O_2, exhibiting a decrease in the first hours followed by an increase after 72 h of stress.

The gene *LOC_Os09g11480* was induced late under *M. grisea* infection, showing no changes at 3 dpi and an increase near to 100-fold at 4 dpi. The gene *LOC_Os01g21120* presented a linear increase along the infection period. Differently of *LOC_Os09g11480*, *LOC_Os02g43790* presented an early induction, showing an increase of nearly 80-fold at 3 dpi, followed by a decrease of expression at 4 dpi.

When one compares the data obtained by microarray (Figure 1) with the ones obtained through qPCR (Figure 3), some differences can be observed in the expression profiles. The genes *LOC_Os01g21120* and *LOC_Os02g43790* showed

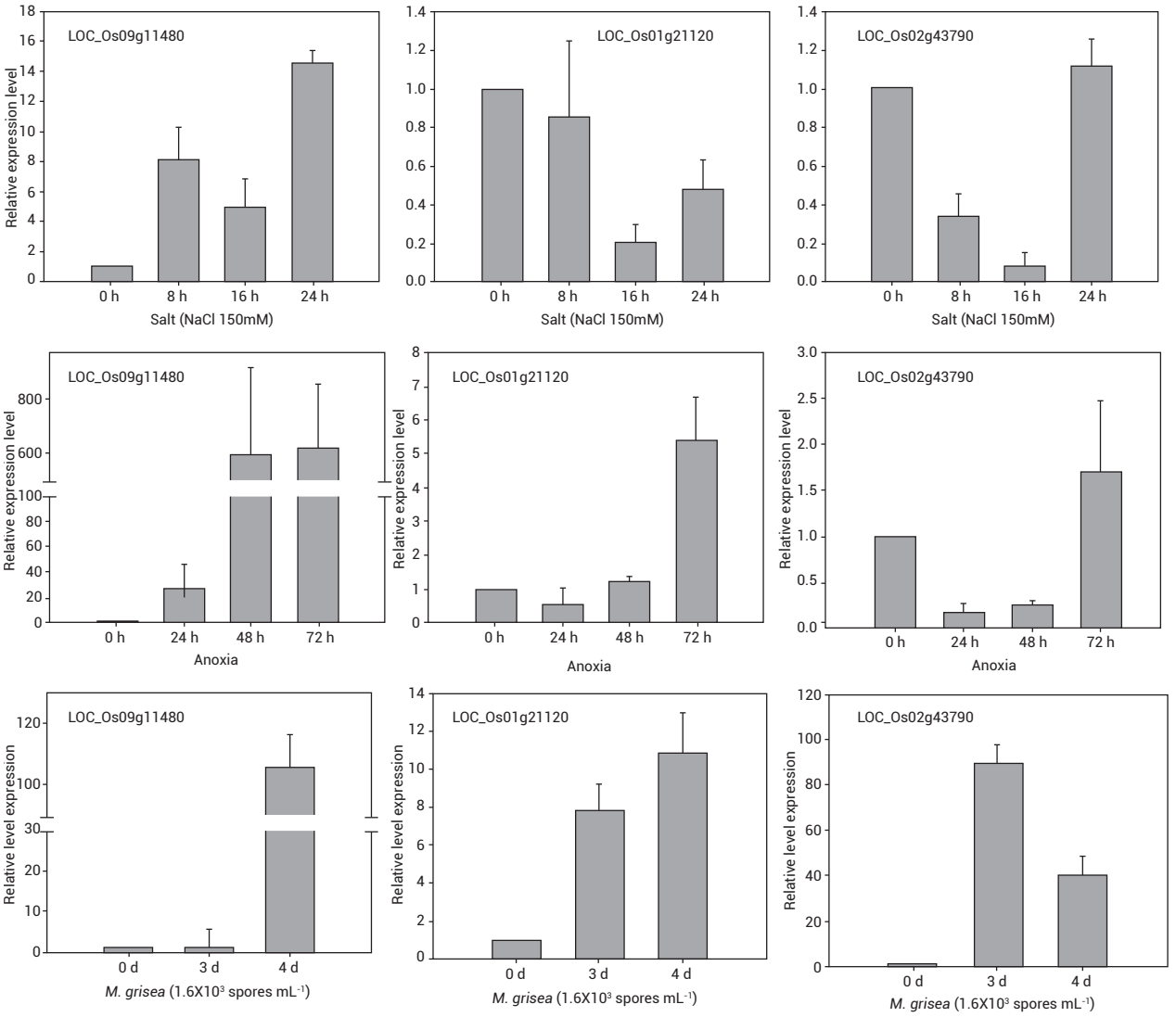

Figure 3. Ethylene response factors gene transcript accumulation in rice, cv. Nipponbare under different stress conditions (salt, anoxia, and *M. grisea*).

similar profile in both techniques, however, *LOC_Os09g11480* did show a contrasting profile. This can be observed when microrrays and qPCR results are compared, thus besides high throughput transcriptomics associated to bioinformatics, it is important to validate these results with more precise techniques and other biological essays.

DISCUSSION

Previous studies suggested that ethylene acts in a complex signalling pathway, composed by phosphorylation cascades and regulation at the transcriptional and post-transcriptional stages (Solano et al. 1998). Interaction of ethylene with other signalling molecules will determine which ethylene responsive genes will be activated in a given cell at a given time, resulting in a wide range of possible responses (Stepanova and Alonso 2009).

Under conditions of complete lack of O_2, there is no ethylene production (Dat et al. 2004). Therefore, the induction of ERF genes under anoxia observed in this work is attributed to other signalling molecules. Among the potential signals for ERF induction, there are reactive oxygen species (ROS) (Wu et al. 2008) formed under anoxia conditions (Cramer et al. 2011). The repression of a large number of ERF can be associated with the inhibition of ethylene production under these conditions.

Besides ethylene production, under salt stress, there is also abscisic acid (Zhang et al. 2006) and ROS (Abogadallah 2010) production. However, in this case, these molecules do not seem to have effect on the regulation of ERF genes.

Overlapping expression in Venn diagrams demonstrate similar response mechanisms in rice ERFs. The overlap of gene expression in response to different stresses has already been reported (Fujita et al. 2006). In the case of the ERF family, this overlap among members can be explained by the fact that some genes can be regulated by different molecules, as ethylene, methyl jasmonate, salycilic and abscisic acids, which are synthesized under different environmental stress conditions (Divi et al. 2010). The overlapping expression of ERFs takes into account the hypothesis that these genes act as connectors in the signalling pathways that mediate responses to biotic and abiotic responses. This mediation has been proven, for instance, in submergence and drought (Fukao et al. 2011).

The unique profiles of ERF found on each stress indicate that these transcription factors are related to the regulation of plant response specifically to that given stress.

The results also suggest sub-functionalization in the ERF gene family, since wherever gene duplicates it can undergo simultaneous reduction of their activity thereby maintaining the total capacity of the ancestral gene (Gallego-Bartolome et al. 2010).

The control of gene expression is critical for determining the stress response mechanism of plants. Many studies have been performed aiming to identify cis acting elements, which play a major role in transcriptional regulation (Walley et al. 2007, Mittal et al. 2009, Zou et al. 2011). However, for the ERF family in rice, very few of such studies have been reported (Jung et al. 2010, Santos et al. 2013).

The cis element with highest occurrence (159) among the ERF genes was CGCGBOXAT (A/C/G)CGCG(G/T/C), which is well known in promoters of genes involved in ethylene, abscisic acid and light perception (Yang and Poovaiah 2002). The second most common cis element (127) was CACTFTPPCA1 (CACT), which is present in the promoter of the C4 isoform of phosphoenolpyruvate carboxylase (Gowik et al. 2004). The third (108) most common element was ACGTATERD1 (ACGT) and is required for induced etiolation (Simpson et al. 2003).

Similarly to what was already mentioned, the increase in expression of ERF genes, observed in anoxia conditions is probably not due to ethylene signalling, but to other molecules, such as ROS. Under other stresses (salt and *M. grisea*), the induction of transcript levels can be associated with the cross-talk of different hormones synthesized under these conditions.

It is possible to conclude that few genes belonging to the ERF gene family present specific expression profiles, while many others are commonly expressed under the different stress conditions studied. Within the ERF family, the regulation of genes is composed of normal (84/115=73%), complex (18/115=16%) and simple regulation (13/115=11%) regarding the presence of different cis elements in the promoter regions. However, weak associations between the presence of specific cis elements and the observed expression profile were detected.

ACKNOWLEDGEMENTS

This work was supported by the Brazilian Ministry of Science and Technology, National Counsel of Technological and Scientific Development (CNPq); Coordination for the Improvement of Higher Education Personnel (CAPES).

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