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RESEARCH ARTICLE

Comparative Analysis of Ethylene Response Factors (ERF1, ERF2) in Three Zoysiagrasses

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Abstract

The APETALA2/ethylene response factor (AP2/ERF) gene family is known to play a key role in the regulating expression of several genes in response to biotic and abiotic stress, conferring an evolutionary advantage to plants. In zoysiagrass, the genetic information of the ERF transcription factors (TF) is limited, leading to gaps in our understanding of the differential response of the zoysiagrasses to external stimuli. In this study, ERF1/2 genes in *Zoysia matrella* and *Z. pacifica* were predicted. We present a comparative study of the ERF1/2 TF among the three Zoysia species, with an overview of their putative gene structures, phylogeny, conserved motifs, and putative protein structure. Our study revealed one ERF1 and ERF2 TF in *Z. pacifica*, but *Z. matrella* had two copies of each ERF1 and ERF2 TF genes. All the ERF1 genes had no introns, while the ERF2 genes exhibited one intron. One AP2/ERF domain was predicted in all the ERF TF genes. Within the AP2 conserved domain of the four *Zoysia* ERF1 genes, a single amino acid substitution (Ala64 → Thr64), as a result of a missense mutation (GCC to ACC) was seen to affect the active ligand- binding site in the GCC-box-domain. The analysis of the upstream regulatory region of the ERF1/2 revealed transcriptional binding motifs linked to regulate the response of ERF genes to abiotic and biotic stresses.

Keywords: Abiotic stress response, Ethylene response factor (ERF1, ERF2), Missense mutation, Protein structure prediction, *Zoysiagrass*

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Introduction

Turfgrasses have been an integral part in the landscaping industry, playing an important part in the urban and suburban environment. The turfgrass industry has a huge impact, both environmentally and economically (Haydu et al., 2006). Among the warm-season turfgrasses, the members of the genera Zoysia are one of the most resilient turfgrasses in terms of their abiotic stress tolerance (Huang et al., 2014). Zoysiagrasses are preferred as turfgrasses because they are salt tolerant, heat and cold tolerant and are aesthetically pleasing (Feng et al., 2019; Patton, 2010). High density genetic linkage mapping has been done in the three zoysiagrasses; *Z. japonica*, *Z. matrella* and *Z. pacifica*, revealing high introgression and hybridization rates (Tanaka et al., 2016). The acquisition of this huge sequencing resource has enabled

researchers to find the molecular mechanisms involved in abiotic and biotic stress tolerance, and to further utilize this information to create better cultivars.

Climate change has initiated a new era of research to identify plants that can thrive in harsh abiotic stress conditions. Unfavorable environmental conditions cause the plants to employ intricate molecular networks involving transcription factors (TFs) that further regulate specific gene expressions, helping the plant cope with stress. One such family of TFs is the APETALA2/ethylene response factor (AP2/ERF) family, characterized by a AP2 DNA binding domain. Their functions range from controlling the developmental and physiological aspects of plant growth to providing defense response in case of abiotic or biotic stress conditions (Shoji and Yuan, 2021). The family can further be divided into AP2, ERF, related to ABI3/VP (RAV), based on the number of AP2 domains and the presence of other DNA binding domains. Members of the subfamily ERF have a single AP2 domain, that specifically binds to GCC-boxes, and are documented to be involved in jasmonate and ethylene signaling and abiotic stress response (Feng et al., 2020).

In *Arabidopsis,* ERF1 has been reported to be highly induced by salt and drought stress (Cheng et al., 2013). In *Z. japonica*, ERF genes (ZjERF1, ZjERF2) have been isolated and are reported to be induced by ethylene, methyl jasmonate and high salinity conditions (Teng et al., 2019). Studies have shown that *Z. matrella* and *Z. pacifica* can regulate tissue salt levels more efficiently than *Z. japonica* (Marcum et al., 1998; Yamamoto et al., 2016). One of the key features in the salt stress tolerance in zoysiagrasses is the presence of salt glands on the leaf surface which help in the secretion of Na^+ (Marcum et al., 1998). So far, no information regarding the ERF genes in *Z. matrella* and *Z. pacifica* has been published to deduce the underlying genetic mechanism of abiotic and biotic stress. The objective of this study is to (i) predict ERF genes in *Z. matrella* and *Z. pacifica*, (ii) Structural and functional characterization of the predicted ERF genes using phylogenetic analysis and protein structure prediction (iii) to identify the probable effects of the variations in the ERF gene among the three zoysiagrasses on the abiotic stress tolerance properties of the zoysiagrasses.

Materials and methods

Sequence data retrieval and identification of *ERF* genes in zoysiagrass

Zoysia japonica (Zj), Zoysia matrella (Zm), and *Zoysia pacifica (Zp)* genome sequences were downloaded from 'Zoysia Genome Database' (http://zoysia.kazusa.or.jp/). The full coding sequence and protein sequence of *Z. japonica* ERF1 and ERF2 was obtained from NCBI (https://www.ncbi.nlm.nih.gov/). This sequence was used as a query for a BLAST (Basic Local Alignment Search Tool) search against *Z. pacifica* and *Z. matrella* genome with the following parameters: expected values ≤1E-5 and more than 80 percent coverage. SNPs and in/dels were identified using SNP-sites (Page et al., 2016). All the BLAST hits were retrieved, and a conserved domain search was performed using NCBI conserved domain search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Multiple alignments of the putative ERF genes were done using ClustalW (https://www.genome.jp/tools-bin/clustalw) (Thompson et al., 1994) with the default settings. Phylogenetic analysis of the ERF transcription factor genes was done using the MEGA X (https://www.megasoftware.net/) maximum likelihood method (Kumar et al., 2018).

Identification of *ERF* upstream regulatory region

The upstream regulatory regions of the *Zoysia ERF* genes were identified by extracting a 1.7 kb sequence upstream of the transcription start site. The putative promoter and TATA box sites were predicted using Transcription Start Sites Plant (TSSP) (Shahmuradov et al., 2017). Further, PlantRegMap (Tian et al., 2020) was used to identify transcription factor binding sites near the

predicted ERF1/2 upstream regulatory regions. Gene Ontology (GO) enrichment analysis was done to identify TF binding sites significantly related to abiotic and biotic stress response signaling (Alexa and Rahnenführer, 2009).

Protein structure prediction and evolutionary conservation analysis

The amino acid sequence of the predicted *Zoysia* ERF genes was used to predict the putative tertiary structure of the proteins. ProtParam tool from Expasy was used to compute the physical and chemical parameters if the predicted proteins (Gasteiger et al., 2005). Protein structure prediction was done using Protein Homology/analogY Recognition Engine 2 (PHYRE^2) (Kelley et al., 2015) and Chimera 1.15 was used for protein structure visualization. The ConSurf server (http://consurf.tau.ac.il/) estimated the conservation pattern of each amino acid in the identified ERF to measure the degree of conservation at each aligned site. The ConSurf scores vary from 1 to 9, with 1 being for fast-evolving (variable) sites. Active ligand binding sites of the proteins were detected using fPocket2 (http://fpocket.sourceforge.net/).

Results and Discussion

Identification of Zoysia ERF transcription factors

The identification of the ERF transcription factors in *Z. matrella* and *Z. pacifica* was done using *Z. japonica* ERF1 and ERF2 full length coding sequences that have been already deposited to the GenBank; accessions MH294481.1 and MH479420.1 respectively. This sequence was used a query for a BLAST search against the *Z. matrella* and *Z. pacifica* genomes. In *Z. matrella*, two sequence hits were obtained for both ERF1 and ERF2 TFs, which were denoted as ZmERF1a/b and ZmERF2a/b respectively (Table 1). The ERF1 genes had no exons, whereas ERF2 genes in all the zoysiagrasses displayed two exons (Fig. 1). Many studies have reported that the number of exons in the ERF gene vary from one to three or more (Ma et al., 2015). The open reading frame in ZmERF1a was 630 bp long encoding 209 amino acids, identical to reports in the ZjERF1 (Teng et al., 2019) . However, ZmERF1b and ZpERF1 had slightly shorter open reading frame of 618bp encoding 205 amino acids. Analysis of SNPs in the predicted ERF1 TFs revealed that both ZmERF1a and ZpERF1 had 6 SNPs and a 12bp deletion with respect to ZjERF1 (Table 2A). Additionally, ZmERF1b had a single nucleotide polymorphism at position 337 (Table 2A). The SNP analysis in the ERF2 genes with respect to the ZjERF1, in the three zoysiagrasses revealed 1, 3 and 5 SNPs in ZmERF2a, ZMERf2b and ZpERF2 respectively (Table 2B). Conserved domain analysis showed that all the ERF genes identified in the zoysiagrasses consisted of one AP2 domain, which is indicative of a typical ERF family transcription factor (Phukan et al., 2017).

Table 1. Zoysia ERF1 and ERF2 predicted genes and their sequence information.

Gene	Code	Gene ID	Strand	Exons	CDS length (bp)	Amino acids
ERF1	*ZjERF1*	Zjn_sc00007.1.g02920.1.sm.mk	+	1	630	209
	ZmERF1a	Zmw_sc03649.1.g00030.1.sm.mk	+	1	630	209
	ZmERF1b	Zmw_sc03126.1.g00090.1.sm.mk	-	1	618	205
	ZpERF1	Zpz_sc01568.1.g00050.1.sm.mk	-	1	618	205
ERF2	*ZjERF2*	Zjn_sc00066.1.g02740.1.am.mk	+	2	729	242
	ZmERF2a	Zmw_sc03147.1.g00050.1.am.mk	-	2	729	242
	ZmERF2b	Zmw_sc05027.1.g00040.1.am.mk	-	2	729	242
	ZpERF2	Zpz_sc00069.1.g00210.1.am.mk	+	2	729	242

ERF: Ethylene responding factor; CDS: Coding sequence; *Zj*: *Z. japonica*; Zm: *Z. matrella*; Zp: *Z. pacifica*.

Table 2. List of SNPs identified with respect to the *Zoysia japonica ERF1/2*.

Item	Position	Ref	Alt	ZmERF1a	ZmERF1b	ZpERF1
A[y]	132	G	A	0	1	1
	135	G	A	0	1	1
	190	G	A	0	1	1
	282	G	C	0	1	1
	288	T	C	0	1	1
	300	T	G	0	1	1
	337	G	C	0	1	0
		Total		0	7	6
B[z]	59	G	A	0	1	1
	294	C	G	0	0	1
	447	G	A	0	1	1
	477	C	T	0	0	1
	671	C	T	1	1	1
		Total		1	3	5

SNP: Single nucleotide polymorphism; Ref: Reference allele; Alt: Alternate allele; Zm: *Z. matrella*; Zp: *Z. pacifica*; *ERF*: Ethylene Response Factor.

[y] SNPs in the *ERF1* genes of *Z. matrella* and *Z. pacifica*.

[z] SNPs in the *ERF2* genes of *Z. matrella* and *Z. pacifica*.

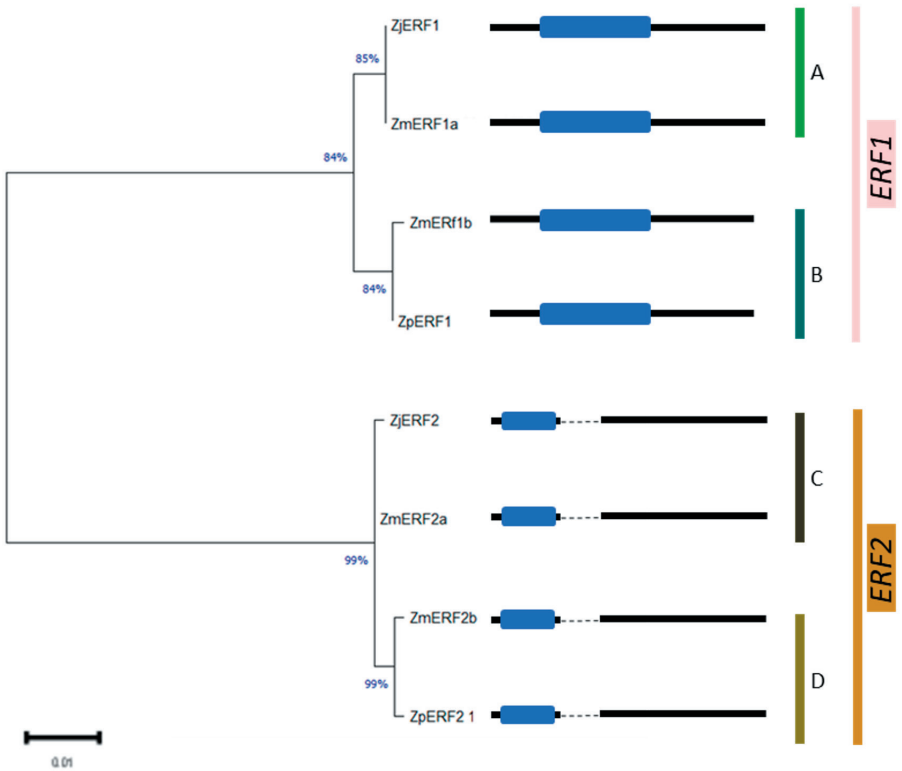

Fig. 1. Phylogenetic relationship of *ERF* transcription factor genes among the three zoysiagrasses using maximum likelihood method with 500 bootstrap replications. The blue boxes represent AP2 domain, black lines represent exons and dotted lines represent introns. ERF: Ethylene Response Factor; AP2: APETALA2; Zj: *Zoysia japonica*; Zm: *Zoysia matrella*; Zp: *Zoysia pacifica*.

Analysis of the upstream regulatory region of the ERF Transcription factor genes

Sequences 1.7 kb upstream of the transcription start site were used to identify putative promoter regions. All ERF1 and ERF2 genes constituted at least one promoter accompanied by a TATA box. The ERF1 predicted promoter region was seen to be highly conserved, whereas the ERF2 promoter region had high variability. In the case of ERF1 in all the three zoysiagrasses constituted one predicted promoter and TATA box site at the same position, with the exception of ZjERF1, having an additional predicted promoter at position -573 and an accompanying TATA box at -544. Each zoysiagrass consisted of two highly conserved promoter regions in the case of ERF2 gene.

Using PlantTFDB (http://plantregmap.gao-lab.org/binding_site_prediction_result.php), top 50 TF binding sites in the 1.7 kb upstream regulatory region (URR) of the transcription start site (TSS) were identified. The results were filtered by removing the duplicates, and only values above q value <0.05 were selected to eliminate false positives. There were more TF binding sites in the URR of the ERF2 gene than in ERF1. Common TF binding sites in both the ERF genes among the three zoysiagrasses included Dof, MIKC_MADS, Trihelix.

GO enrichment analysis was conducted to identify the TF binding sites related to the documented ERF TFs functions. The ERF1 TF binding sites in the URR revealed many motifs, that were related to responses to plant development and flower regulation, responses to plant hormones and biotic and abiotic stresses. The motif Zjn_sc00045.1.g02180.1.sm.mk in ZjERF1, belonging to Dof family, engages in a wide range of responses like response to drought salinity plant hormones, bacteria, and fungi. The motifs Zjn_sc00007.1.g03660.1.sm.mk and Zmw_sc01431.1.g00150.1 belonging to ERF family were related to response to cold and heat stress, and response to salicylic acid and jasmonic acid. The TF binding sites in *Z. pacifica* ERF1 were related to floral organ identity, flower development fruit development. Motifs related to response to abiotic and biotic stresses were absent in the ZpERF1 URR (Table S1).

Transcription factor binding sites in the ERF2 URR of the four genes were related to the WRKY, MIKC_MADS, HD-ZIP and MYB_related TF families (Table S2). The motifs Zjn_sc00024.1.g02600.1.sm.mkhc and Zjn_sc00075.1.g00940.1.am.mkhc in the ZjERF2 URR belong to the WRKY family and are involved jasmonic acid mediated pathway, response to salicylic acid and defense response to fungal and bacterial attack. The ZmERF2a,b URRs also consisted of motifs belonging to the WRKY TF family, which respond to salicylic acid, chitin, and fungal and bacterial attack. The motifs belonging to HD-ZIP family in ZmERF2a,b (Zmw_sc00963.1.g00120.1, Zmw_sc00963.1.g00120.1), may have a role in salt stress or osmotic stress response. In *Z. pacifica* ERF2, motifs belonging to the WRKY family (Zpz_sc00228.1.g00240.1.sm.mk, Zpz_sc02744.1.g00050.1.sm.mkhc, Zpz_sc00990.1.g00040.1.sm.mkhc) may respond to jasmonic acid stimulus, and fungal and bacterial attacks. The motif Zpz_sc00747.1.g00070.1.sm.mk, belonging to WRKY family may be involved in the negative regulation of gibberellic acid pathway.

Most of the TF binding sites had a redundant function, specifically in the two copies of the *Z. matrella* ERF1 and ERF2 transcription factors. Such redundant TF clusters may indicate gene regulatory hotspots (Dergilev et al., 2022).

Phylogenetic relation of the ERF gene in the three Zoysiagrasses

To determine the evolutionary relationship among the ERF genes, an unrooted phylogenetic tree based on the maximum likelihood method was constructed using MEGA X based on the multiple sequence alignment of the 8 ERF transcription factor genes. The phylogenetic tree showed that ERF1 and ERF2 were distinctly separated into two major groups, which were further divided into two subgroups. ZmERF1a was found to be closely related to ZjERF1 (subgroup A), whereas ZmERF1b and ZpERF1 were grouped together (subgroup B) (Fig. 1). In the ERF2 group, further two subgroups were formed with ZjERF2 and ZmERF2a forming subgroup C and ZmERF2b and ZpERF2 grouped together in subgroup D (Fig. 1).

Evolutionary-based conservation analysis of the predicted ERF1/2 proteins

The ERF1/2 amino acid sequences obtained from the three zoysiagrasses were aligned using ClustalW. The amino acids in the AP2 binding domain of the ERF1 protein were marked as highly conserved by ConSurf. Within the AP2 conserved domain, one amino acid substitution was observed at position 64, wherein the Alanine in ZjERF1 and ZmERF1 is substituted with Threonine in ZmERF1b and ZpERF1 (Fig. 2A). This missense mutation is a result of a single base substitution in the codon: GCC (producing alanine) to ACC (producing threonine). Both the amino acids were seen to be categorized as evolutionary conserved in the ConSurf analysis, implying that the amino acid changes at the position 64 may affect the structure or function of the protein drastically. Outside the AP2 conserved domain, another amino acid substitution was seen at position 113, where Aspartic acid was substituted by Histidine in ZmERF1b. The amino acids at the position 113 were categorized as highly variable by ConSurf analysis, leading to a conclusion that they may have little to no effect the properties of the ERF1 protein.

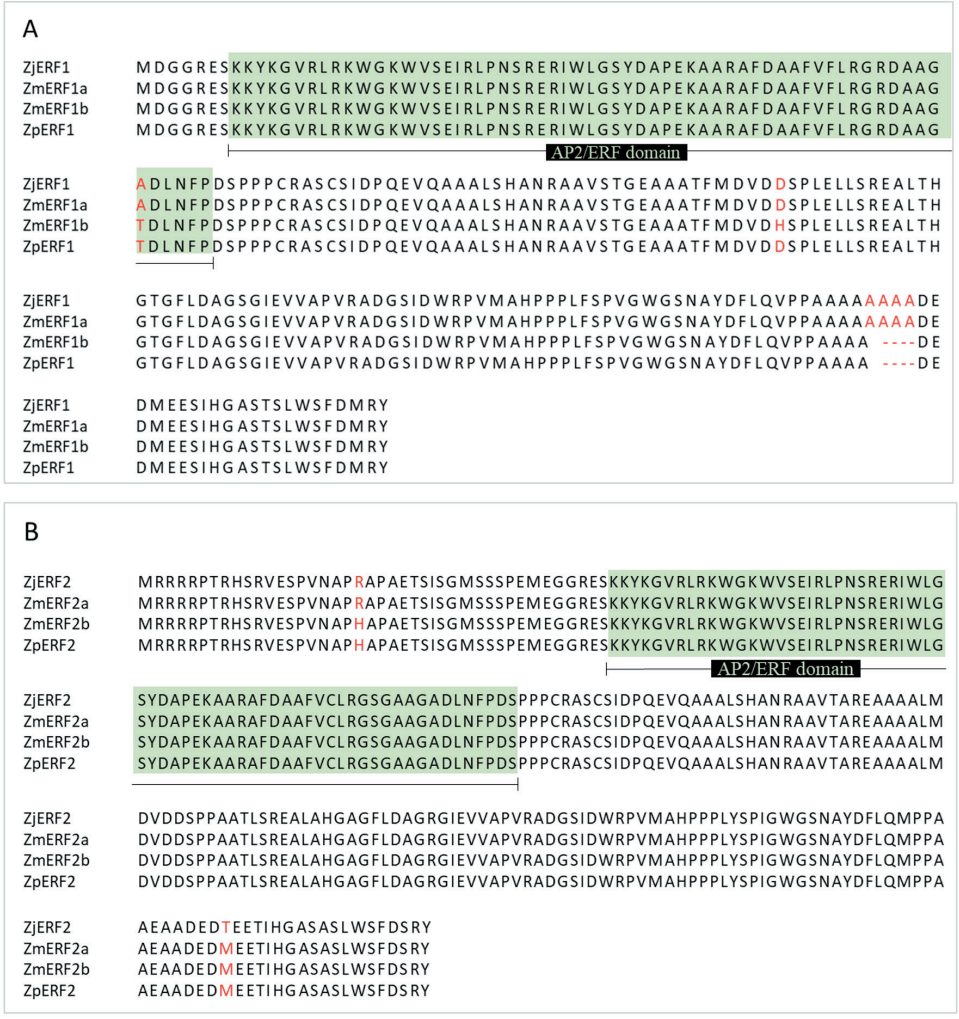

Fig. 2. Conserved domain analysis of the *Zoysia*. (A) ERF1 putative protein sequences and (B) ERF2 putative protein sequences. ERF proteins are characterized by one AP2/ERF domain (highlighted in green). The single amino acid polymorphic sites and in/del sites are in red. ERF: Ethylene Response Factor; AP2: APETALA2; Zj: *Zoysia japonica*; Zm: *Zoysia matrella*; Zp: *Zoysia pacifica*.

The ERF1 and ERF2 protein structures were highly disordered (60-62%) in all the zoysiagrasses. Intrinsically disordered proteins (IDPs) make up almost 30-50% of the eukaryotic proteins. These proteins are biologically active, but are unable to adopt persistent tertiary structures, leading to dynamic conformational ensembles (Salladini et al., 2020). The AP2/ERF family domain binding sites are flanked with intrinsically disordered residues, lacking secondary structures. ZjERF1, ZmERF1a and ZpERF1 protein constituted of 24% of the structure forming alpha helices and the 9% forming beta sheets (Fig. 3). ZmERF1b had the lowest structural disorder (57%) among the other zoysia ERF1 proteins, with a structure consisting of 24% alpha helices and 9% beta sheets (Fig. 3). ZjERF2 and ZmERF2a protein structures constituted 30% alpha helices and 7% beta sheets, whereas ZmERF2b and ZpERF2 protein structures constituted 28% alpha helices and 5% beta sheets (Fig. 4).

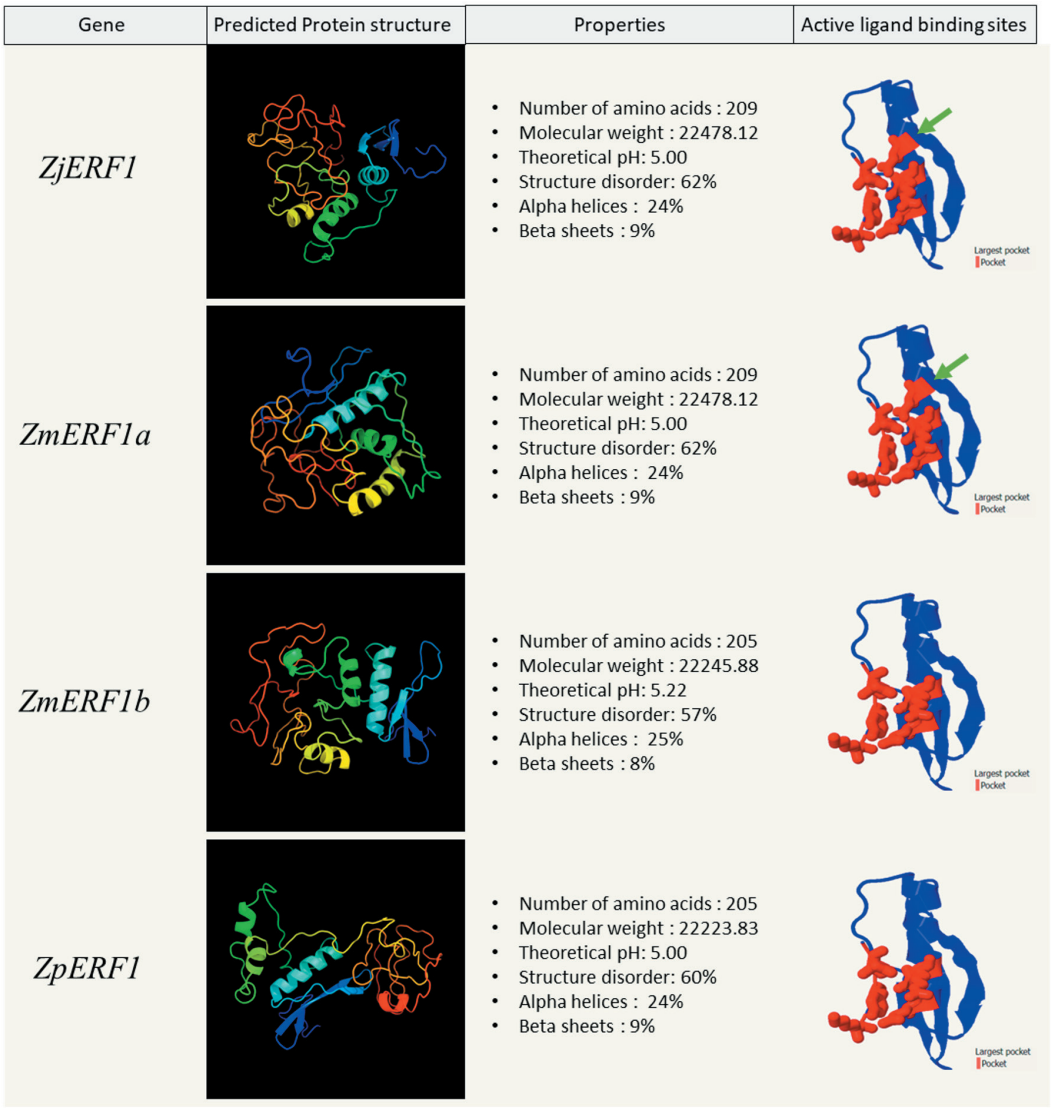

Fig. 3. *Zoysia* ERF1 proteins in the physical, chemical, and structural analysis. For each ERF1 protein, the protein structure was predicted using PHYRE2 (image colored by rainbow N to C terminus). Largest active ligand binding sites (in red) were predicted using fPocket2. Green arrows denote an additional active binding site in ZjERF1 and ZmERF2a. ERF: Ethylene Response Factor; Zj: *Zoysia japonica*; Zm: *Zoysia matrella*; Zp: *Zoysia pacifica*.

Protein homology search of the ERF1 and ERF2 predicted sequences yielded a significant hit to the GCC-box binding domain, which is a core function of the ERF transcription factors (Zarei et al., 2011). Protein pocket detection can reveal the functional & ligand binding sites in a protein (Stank et al., 2016). The detection of active ligand binding sites in the GCC-box binding domain of the ERF proteins was done using fPocket2. The active pocket of ZjERF1 and ZmERF1a were identical whereas the pockets of ZmERF1b and ZpERF1 were identical to each other (Fig. 3). The difference among these two groups was seen as a result of an amino acid substitution at position 64 (Alanine → Threonine) in the AP2/ERF domain (Fig. 2). The active binding site in the GCC-box-domain of all the four ERF2 TFs was identical, owing to the fact that there were no amino acid substitutions seen in the conserved AP2/ERF domain (Fig. 4).

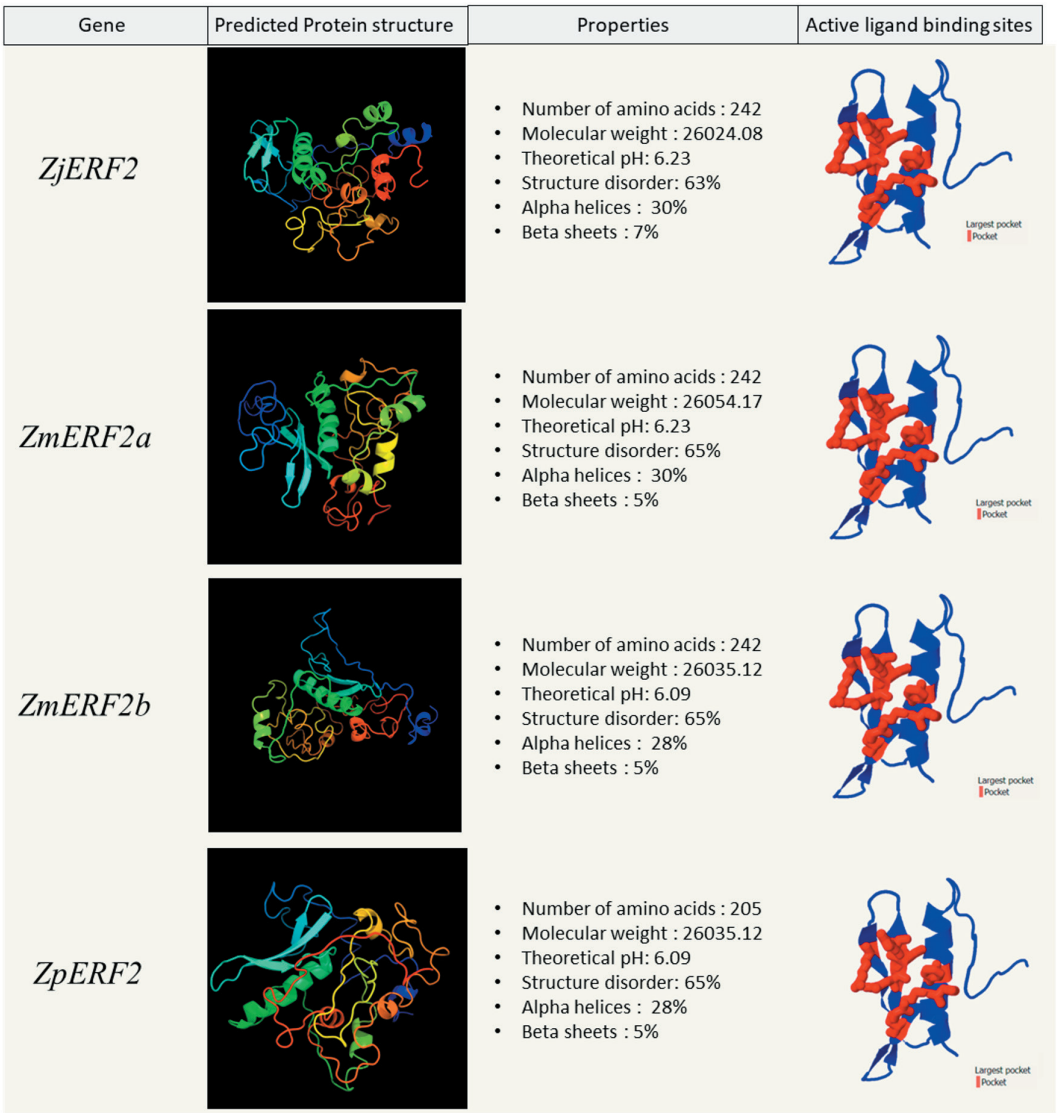

Fig. 4. *Zoysia* ERF2 proteins physical, chemical, and structural analysis. For each ERF2 protein, the protein structure was predicted using PHYRE2 (image colored by rainbow N to C terminus) . Largest active ligand binding sites (in red) were predicted using fPocket2. ERF: Ethylene Response Factor; Zj: *Zoysia japonica*; Zm: *Zoysia matrella*; Zp: *Zoysia pacifica*.

Conclusion

In the wake of climate change, zoysiagrasses have recently come under the spotlight to explore their genomes and identify genes that confer better survival in adverse environmental conditions. Members of the zoysiagrasses like *Z. japonica* have been widely studied for its cold and drought tolerance (Cohen et al., 2019; Kaur et al., 2022). Studies on the salt tolerance mechanism in the zoysiagrasses revealed that *Z. matrella* and *Z. pacifica* have a higher salt tolerance capability than *Z. japonica* (Loch et al., 2005; Marcum et al., 1998; Sugiura and Takahashi, 2021). The varying degrees of salt tolerance among the zoysiagrasses was attributed to leaching (Loch et al., 2005) , but the key genetic players behind the salt tolerance capability have been poorly studied. *Z. japonica* , *Z. matrella* and *Z. pacifica show* poor drought tolerance capabilities when compared to Bermudagrass (Loch et al., 2017). ERF gene family is an excellent candidate to study the differential regulation of genetic mechanisms that confer biotic and abiotic stress resistance in plants (Owji et al., 2017).

The present study aimed to predict the ERF1 and ERF2 TF genes in *Z. matrella* and *Z. pacifica* and to compare the probable effects of the variation in the gene structure among the Zoysia grasses. Phylogenetic analysis divided the eight ERF TFs to two groups, which was consistent with the previous studies where the basis of classification was the presence of introns and conserved motifs (Ma et al., 2015). The phylogenetic grouping was further validated by the protein structure analysis, wherein the ERF TF genes belonging to the same subgroups has similar structure and chemical properties. The ERF TFs are known to be involved in hormonal, abiotic stress and biotic stress signaling pathways (Shoji and Yuan, 2021). The TF binding motifs in the upstream regulatory region of each ERF gene had at least one motif related to these signaling pathways, with the exception of ZpERF1 having no significant motifs related to abiotic or biotic stress signaling. The effect of the single amino acid substitution at the 64th amino acid between the sub-group A (ZjERF1, ZmERF1a) and sub-group B (ZmERF1b, ZpERF1) was seen in the analysis of the GCC-box-binding domain, wherein the presence of Alanine in sub-group A conferred an additional active site. The experimental validation of the effect of Ala64 to Thr64 substitution can provide some clues in understanding the variation in the abiotic stress response among the three zoysiagrasses. The probable effect of the TF binding motifs in the upstream regulatory regions of the ERF transcription start site may also be studied further to identify their roles in enhancing stress signaling mechanisms for in *Zoysia*. The information provided in this study aims to add to the otherwise scarce resource of the zoysiagrass research pool.

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Table S1. List of transcription factor binding sites in the upstream regulatory region of the *Zoysia ERF1* gene.

Gene name	Pattern name	Family	*P*-value	*q*-value	Matched sequence
ZjERF1	Zjn_sc00022.1.g03830.1.sm.mkhc	Dof	2.43E-07	0.0008	AACAAAGTAAAAAAGATAAAA
	Zjn_sc00045.1.g02180.1.sm.mk	MYB	7.61E-07	0.0025	TAGTCGGTTTTTATAGTTT
	Zjn_sc00007.1.g09020.1.sm.mk	MIKC_MADS	2.79E-06	0.0083	TTGCCGAAATGGGCAAGAT
	Zjn_sc00036.1.g04050.1.am.mk	C2H2	2.80E-06	0.009	ATGCCCTATTTGTCCTTTTCT
	Zjn_sc00008.1.g01280.1.am.mkhc	G2-like	4.27E-06	0.014	AAGAAAACA
	Zjn_sc00020.1.g00610.1.am.mk	Trihelix	7.40E-06	0.0183	ATTTCTCCGGCCAGA
	Zjn_sc00007.1.g03660.1.sm.mk	ERF	7.40E-06	0.0247	CCACCACTGTGCCCACCGTC
	Zjn_sc00003.1.g11150.1.sm.mkhc	Dof	8.21E-06	0.026	CAAAGTAAAAAAGATAAAA
ZmERF1a	Zmw_sc02636.1.g00060.1	Dof	2.43E-07	0.0008	AACAAAGTAAAAAAGATAAAA
	Zmw_sc04298.1.g00070.1	Dof	2.43E-07	0.0008	AACAAAGTAAAAAAGATAAAA
	Zmw_sc00834.1.g00100.1	MIKC_MADS	2.79E-06	0.0082	TTGCCGAAATGGGCAAGAT
	Zmw_sc00641.1.g00070.1	MIKC_MADS	2.79E-06	0.0083	TTGCCGAAATGGGCAAGAT
	Zmw_sc01702.1.g00160.1	Trihelix	7.40E-06	0.0183	ATTTCTCCGGCCAGA
	Zmw_sc01431.1.g00150.1	ERF	7.40E-06	0.0247	CCACCACTGTGCCCACCGTC
	Zmw_sc00508.1.g00150.1	Dof	8.21E-06	0.0261	CAAAGTAAAAAAGATAAAA
	Zmw_sc00722.1.g00320.1	WRKY	1.19E-05	0.0383	CAAAGTCAAAC
	Zmw_sc05484.1.g00050.1	GATA	1.42E-05	0.0454	CACCGTCGTTGGCATCATT
ZmERF1b	Zmw_sc07405.1.g00010.1	Dof	2.53E-09	0.0022	TTTTCTTTTTTACTTTGTTTT
	Zmw_sc01471.1.g00360.1	Dof	1.92E-08	0.0012	ATTTTTTTCTTTTTTACTTTGTTTTTATAT
	Zmw_sc03232.1.g00050.1	HD-ZIP	4.59E-07	0.0013	TAACCAATAATTACTGTATTT
	Zmw_sc02637.1.g00050.1	C2H2	9.82E-07	0.0029	GAGAAAAGGACAAATAG
	Zmw_sc02674.1.g00130.1	C2H2	1.98E-06	0.0031	GAGAAAAGGACAAATAG
	Zmw_sc01968.1.g00070.1	C2H2	2.46E-06	0.0039	GAGAAAAGGACAAATAGG
	Zmw_sc00175.1.g00130.1	C2H2	2.46E-06	0.004	GAGAAAAGGACAAATAGG
	Zmw_sc02375.1.g00060.1	C2H2	2.43E-06	0.0079	AGAAAAGGACAAATA
	Zmw_sc05144.1.g00030.1	MIKC_MADS	4.86E-06	0.0079	GTTTTCCATTGTTTTCTTTTG
	Zmw_sc01547.1.g00050.1	Trihelix	2.93E-06	0.0088	TTTTTTACTTTGTT
	Zmw_sc01893.1.g00110.1	C2H2	6.16E-06	0.01	GAGAAAAGGACAAATAGGGC
	Zmw_sc00553.1.g00060.1	ZF-HD	4.40E-06	0.0133	TAACCAATAATTAC
	Zmw_sc00067.1.g00200.1	ZF-HD	4.40E-06	0.0134	TAACCAATAATTAC
	Zmw_sc02938.1.g00100.1	MIKC_MADS	5.43E-06	0.017	TCTTGCCTATTTCGG
	Zmw_sc01492.1.g00030.1	G2-like	5.86E-06	0.0184	ACAAGATACGCTGG
	Zmw_sc03476.1.g00010.1	CPP	2.58E-05	0.0306	ATTACTGTATTTAAA
	Zmw_sc04298.1.g00070.1	Dof	2.05E-05	0.0319	AGAGGATTCGTAAAGTAGAAA
	Zmw_sc02636.1.g00060.1	Dof	2.05E-05	0.0319	AGAGGATTCGTAAAGTAGAAA
	Zmw_sc05692.1.g00060.1	NAC	1.11E-05	0.0371	GAACAAGCCAAG
	Zmw_sc01394.1.g00160.1	MIKC_MADS	1.17E-05	0.0377	TTGCCTATTTCGGCA
	Zmw_sc01933.1.g00080.1	MYB	4.26E-05	0.0441	TGAAATTGGTTAGTTAAAATT
ZpERF1	Zpz_sc00110.1.g00170.1.am.mk	Dof	9.23E-08	0.0003	AACAAAGTAAAAAAGAAAAAA
	Zpz_sc01065.1.g00130.1.am.mk	Trihelix	1.48E-07	0.0004	AAAGTAAAAAAGAAAA
	Zpz_sc00142.1.g00660.1.sm.mk	MIKC_MADS	6.43E-07	0.0019	TTGCCGAAATAGGCAAGAT
	Zpz_sc01322.1.g00090.1.sm.mkhc	C2H2	6.13E-07	0.0019	AAGAATACGACAAATGA
	Zpz_sc00459.1.g00330.1.am.mk	C2H2	7.00E-07	0.0023	TATTTGTCCTTTTCTC
	Zpz_sc01822.1.g00030.1.am.mk	C2H2	1.99E-06	0.004	AAGAATACGACAAATGAC
	Zpz_sc00060.1.g00790.1.sm.mkhc	Dof	4.06E-06	0.013	CAAAGTAAAAAAGAAAAAA
	Zpz_sc02813.1.g00030.1.sm.mk	ERF	7.40E-06	0.0248	CCACCACTGTGCCCACCGTC
	Zpz_sc02621.1.g00020.1.am.mk	Trihelix	7.40E-06	0.0249	ATTTCTCCGGCCAGA
	Zpz_sc01918.1.g00050.1.am.mk	ERF	1.06E-05	0.0354	CACCACTGTGCCCACCGTCG
	Zpz_sc01479.1.g00080.1.am.mk	MIKC_MADS	1.28E-05	0.0417	AGTAAAAAAGAAAA
	Zpz_sc02943.1.g00100.1.am.mk	MIKC_MADS	1.28E-05	0.0418	AGTAAAAAAGAAAA

ERF: Ethylene responding factor; *Zj*: *Z. japonica*; Zm: *Z. matrella*; Zp: *Z. pacifica*.

Table S2. List of transcription factor binding sites in the upstream regulatory region of the *Zoysia ERF2* gene. (continued)

Gene name	Pattern name	Family	*P*-value	*q*-value	Matched sequence
ZjERF2	Zjn_sc00002.1.g03010.1.es.mkhc	G2-like	9.42E-07	0.00147	TATAGAATATTTTT
	Zjn_sc00159.1.g00110.1.sm.mk	WRKY	7.49E-07	0.00202	ACAGTTGACTTTTA
	Zjn_sc00007.1.g09020.1.sm.mk	MIKC_MADS	1.53E-06	0.0025	TTACTAAATATAGAAATAG
	Zjn_sc00024.1.g02600.1.sm.mkhc	WRKY	1.70E-06	0.00263	AGTTGACTTTTAA
	Zjn_sc00075.1.g00940.1.am.mkhc	WRKY	9.09E-07	0.00269	ACAGTTGACTTTT
	Zjn_sc00009.1.g04440.1.sm.mk	MIKC_MADS	1.34E-06	0.0039	AAAAAAAAAGAAAA
	Zjn_sc00068.1.g02930.1.sm.mk	MIKC_MADS	1.95E-06	0.00452	TACTAAATATAGAAA
	Zjn_sc00133.1.g00490.1.sm.mkhc	WRKY	1.44E-06	0.00459	ACAGTTGACTTTT
	Zjn_sc00008.1.g04060.1.sm.mkhc	WRKY	3.72E-06	0.00552	AAGTTTGACTTTTAC
	Zjn_sc00022.1.g04290.1.sm.mkhc	G2-like	3.70E-06	0.00582	AATAGAATATTATT
	Zjn_sc00022.1.g03830.1.sm.mkhc	Dof	2.87E-06	0.00663	CATCAAAAAAAAAAGAAAAAC
	Zjn_sc00003.1.g14360.1.sm.mkhc	MIKC_MADS	9.00E-06	0.00888	GCTCAGTTTTTTTTTTCCTCC
	Zjn_sc00043.1.g05100.1.am.mk	WRKY	3.25E-06	0.00917	AGTTGACTTTTAATAGTAT
	Zjn_sc00012.1.g01260.1.am.mk	WRKY	3.61E-06	0.0101	ATATTAACACAGTTGACTTTT
	Zjn_sc00036.1.g04050.1.am.mk	C2H2	4.16E-06	0.0115	TATTTGTTGTTGTCTTTGTCC
	Zjn_sc00028.1.g04380.1.sm.mk	G2-like	4.20E-06	0.0116	AATAGAATATT
	Zjn_sc00011.1.g02540.1.sm.mk	WRKY	5.30E-06	0.0146	CAGTTGACTTTTAA
	Zjn_sc00003.1.g11150.1.sm.mkhc	Dof	8.59E-06	0.0157	ATATAAAAAAAAGTATACT
	Zjn_sc00075.1.g00920.1.am.mk	WRKY	5.68E-06	0.0166	ACAGTTGACTTT
	Zjn_sc00011.1.g04160.1.am.mkhc	MYB	9.03E-06	0.0171	TAGAAATATGATAAAAATATG
	Zjn_sc00025.1.g03620.1.sm.mk	MYB	9.03E-06	0.0173	TAGAAATATGATAAAAATATG
	Zjn_sc00022.1.g02500.1.sm.mkhc	HD-ZIP	1.06E-05	0.02	AAAATAATTGT
	Zjn_sc00003.1.g14950.1.am.mk	Trihelix	1.06E-05	0.0208	ATTTTTACCTCAAA
	Zjn_sc00034.1.g05970.1.am.mk	Dof	1.11E-05	0.0272	CAAAAAGTAAT
	Zjn_sc00069.1.g01520.1.sm.mkhc	C3H	1.11E-05	0.0275	AAAAAAAAAGAAAA
	Zjn_sc00034.1.g00090.1.sm.mk	GATA	1.15E-05	0.028	GATGTTCTTGACTGTCAAAATCATTAG
	Zjn_sc00011.1.g03120.1.sm.mkhc	GATA	1.11E-05	0.0335	GATATAGATCTAT
	Zjn_sc00067.1.g02160.1.sm.mkhc	BBR-BPC	2.10E-05	0.0345	GAATATTTCTCTCTCTTACAA
ZmERF2a	Zmw_sc07168.1.g00040.1	G2-like	9.42E-07	0.00148	TATAGAATATTTTT
	Zmw_sc00179.1.g00320.1	WRKY	1.70E-06	0.00261	AGTTGACTTTTAA
	Zmw_sc00823.1.g00100.1	WRKY	1.01E-06	0.00269	GCAGTTGACTTTTA
	Zmw_sc02214.1.g00030.1	WRKY	1.07E-06	0.00314	GCAGTTGACTTTT
	Zmw_sc03476.1.g00010.1	CPP	2.59E-06	0.00494	TATTTCATATTTAAA
	Zmw_sc03239.1.g00050.1	WRKY	2.11E-06	0.00541	GCAGTTGACTTTT
	Zmw_sc04648.1.g00070.1	G2-like	3.70E-06	0.00586	AATAGAATATTATT
	Zmw_sc01245.1.g00180.1	WRKY	3.72E-06	0.00604	AAGTTTGACTTTTAC
	Zmw_sc03298.1.g00030.1	WRKY	3.25E-06	0.00921	AGTTGACTTTTAATAGTAT
	Zmw_sc06259.1.g00020.1	WRKY	3.56E-06	0.00991	ATATTAACGCAGTTGACTTTT
	Zmw_sc02449.1.g00110.1	G2-like	4.20E-06	0.0114	AATAGAATATT
	Zmw_sc05892.1.g00030.1	G2-like	4.20E-06	0.0115	AATAGAATATT
	Zmw_sc04801.1.g00080.1	WRKY	5.30E-06	0.0147	CAGTTGACTTTTAA
	Zmw_sc00039.1.g00390.1	WRKY	5.25E-06	0.0154	GCAGTTGACTTT
	Zmw_sc00508.1.g00150.1	Dof	8.59E-06	0.0156	ATATAAAAAAAAGTATACT
	Zmw_sc01004.1.g00150.1	Dof	1.17E-05	0.0179	CAAAAAGTAA
	Zmw_sc04272.1.g00050.1	HD-ZIP	9.78E-06	0.0196	ACATTAAATAT
	Zmw_sc00963.1.g00120.1	HD-ZIP	1.06E-05	0.02	AAAATAATTGT
	Zmw_sc01547.1.g00050.1	Trihelix	1.06E-05	0.0208	ATTTTTACCTCAAA
	Zmw_sc02388.1.g00110.1	MYB	2.09E-05	0.0224	AGAAAATAAGATAAAAAGTTT
	Zmw_sc02636.1.g00060.1	Dof	1.10E-05	0.0233	CAATATAAAAAAAAGTATACT
	Zmw_sc04298.1.g00070.1	Dof	1.10E-05	0.0236	CAATATAAAAAAAAGTATACT
	Zmw_sc00431.1.g00450.1	Dof	1.11E-05	0.0269	CAAAAAGTAAT
	Zmw_sc01579.1.g00050.1	Dof	1.11E-05	0.0271	CAAAAAGTAAT
	Zmw_sc00326.1.g00320.1	BBR-BPC	2.10E-05	0.0278	GAATATTTCTCTCTCTTACAA
	Zmw_sc05134.1.g00060.1	GATA	1.15E-05	0.0283	GATGTTCTTGACTGTCAAAATCATTAG
	Zmw_sc01492.1.g00150.1	GATA	1.15E-05	0.0283	GATGTTCTTGACTGTCAAAATCATTAG
	Zmw_sc00711.1.g00180.1	GATA	1.11E-05	0.0334	GATATAGATCTAT
	Zmw_sc03046.1.g00040.1	WRKY	1.45E-05	0.0492	CGGTCAAC

Table S2. List of transcription factor binding sites in the upstream regulatory region of the *Zoysia ERF2* gene.

Gene name	Pattern name	Family	*p*-value	*q*-value	Matched sequence
ZmERF2b	Zmw_sc00823.1.g00100.1	WRKY	7.49E-07	0.00197	ACAGTTGACTTTTA
	Zmw_sc00179.1.g00320.1	WRKY	1.70E-06	0.00225	AGTTGACTTTTAA
	Zmw_sc02214.1.g00030.1	WRKY	9.09E-07	0.00263	ACAGTTGACTTTT
	Zmw_sc07168.1.g00040.1	G2-like	1.26E-06	0.00288	AATAGAATATTATT
	Zmw_sc03239.1.g00050.1	WRKY	1.44E-06	0.00418	ACAGTTGACTTTT
	Zmw_sc01245.1.g00180.1	WRKY	3.91E-06	0.00789	ACAGTTGACTTTTAA
	Zmw_sc03298.1.g00030.1	WRKY	3.25E-06	0.00906	AGTTGACTTTTAATAGTAT
	Zmw_sc04648.1.g00070.1	G2-like	3.70E-06	0.0091	AATAGAATATTATT
	Zmw_sc06259.1.g00020.1	WRKY	3.61E-06	0.00984	ATATTAACACAGTTGACTTTT
	Zmw_sc05892.1.g00030.1	G2-like	4.20E-06	0.0113	AATAGAATATT
	Zmw_sc02449.1.g00110.1	G2-like	4.20E-06	0.0113	AATAGAATATT
	Zmw_sc04801.1.g00080.1	WRKY	5.30E-06	0.0144	CAGTTGACTTTTAA
	Zmw_sc00508.1.g00150.1	Dof	8.59E-06	0.015	ATATAAAAAAAAGTATACT
	Zmw_sc03299.1.g00040.1	NAC	5.95E-06	0.0162	TACGTATTCGATTTAGATATCT
	Zmw_sc00039.1.g00390.1	WRKY	5.68E-06	0.0166	ACAGTTGACTTT
	Zmw_sc00860.1.g00250.1	Trihelix	6.46E-06	0.0168	TACATGTGTTTAAAGTT
	Zmw_sc02388.1.g00110.1	MYB	1.13E-05	0.0174	TAGAAATATGATAAAAATATT
	Zmw_sc01004.1.g00150.1	Dof	1.17E-05	0.0175	CAAAAAGTAA
	Zmw_sc00963.1.g00120.1	HD-ZIP	1.06E-05	0.019	AAAATAATTGT
	Zmw_sc04272.1.g00050.1	HD-ZIP	9.78E-06	0.0193	ACATTAAATAT
	Zmw_sc01547.1.g00050.1	Trihelix	1.06E-05	0.02	ATTTTTACCTCAAA
	Zmw_sc00463.1.g00070.1	G2-like	2.02E-05	0.0201	AATAGAATATTATTT
	Zmw_sc01160.1.g00110.1	MYB_related	8.95E-06	0.0216	AGATATTTAA
	Zmw_sc01754.1.g00080.1	MYB_related	1.09E-05	0.0225	AGATATTTAAA
	Zmw_sc00868.1.g00150.1	SBP	7.39E-06	0.023	TCCGTACAGCT
	Zmw_sc01579.1.g00050.1	Dof	1.11E-05	0.0261	CAAAAAGTAAT
	Zmw_sc00431.1.g00450.1	Dof	1.11E-05	0.0262	CAAAAAGTAAT
	Zmw_sc00711.1.g00180.1	GATA	1.11E-05	0.0329	GATATAGATCTAT
	Zmw_sc03149.1.g00140.1	CPP	5.38E-05	0.0358	TATTTAAATT
	Zmw_sc00911.1.g00130.1	HD-ZIP	3.99E-05	0.0389	AAAATAATTGT
	Zmw_sc01382.1.g00110.1	YABBY	3.50E-05	0.0396	TATGATAA
	Zmw_sc01000.1.g00270.1	YABBY	3.50E-05	0.0396	TATGATAA
	Zmw_sc00909.1.g00270.1	MYB_related	1.75E-05	0.042	CAGATATTT
	Zmw_sc03046.1.g00040.1	WRKY	1.45E-05	0.0492	CGGTCAAC
ZpERF2	Zpz_sc00747.1.g00070.1.sm.mk	WRKY	7.49E-07	0.00198	ACAGTTGACTTTTA
	Zpz_sc00228.1.g00240.1.sm.mk	WRKY	1.70E-06	0.00225	AGTTGACTTTTAA
	Zpz_sc02744.1.g00050.1.sm.mkhc	WRKY	9.09E-07	0.00265	ACAGTTGACTTTT
	Zpz_sc00885.1.g00020.1.es.mkhc	G2-like	1.26E-06	0.00291	AATAGAATATTATT
	Zpz_sc01607.1.g00140.1.sm.mkhc	WRKY	1.44E-06	0.00418	ACAGTTGACTTTT
	Zpz_sc01065.1.g00130.1.am.mk	Trihelix	4.44E-06	0.00594	ATAGTAAATATATATA
	Zpz_sc00990.1.g00040.1.sm.mkhc	WRKY	3.91E-06	0.00788	ACAGTTGACTTTTAA
	Zpz_sc00459.1.g00330.1.am.mk	C2H2	2.93E-06	0.00886	TTGTTGTCTTTATCTG
	Zpz_sc00389.1.g00020.1.am.mk	WRKY	3.25E-06	0.00899	AGTTGACTTTTAATAGTAT
	Zpz_sc00890.1.g00190.1.sm.mk	G2-like	4.20E-06	0.0112	AATAGAATATT
	Zpz_sc00657.1.g00170.1.am.mk	MYB	5.64E-06	0.0135	TTTATAGTTTGTTCAGTTT
	Zpz_sc00289.1.g00210.1.am.mk	WRKY	5.30E-06	0.0145	CAGTTGACTTTTAA
	Zpz_sc00663.1.g00060.1.sm.mkhc	NAC	5.95E-06	0.0162	TACGTATTCGATTTAGATATCT
	Zpz_sc06143.1.g00010.1.am.mk	WRKY	5.68E-06	0.0166	ACAGTTGACTTT
	Zpz_sc02744.1.g00030.1.am.mk	WRKY	5.68E-06	0.0166	ACAGTTGACTTT
	Zpz_sc02347.1.g00040.1.am.mk	MYB	1.13E-05	0.0174	TAGAAATATGATAAAAATATT
	Zpz_sc00452.1.g00080.1.am.mk	HD-ZIP	9.78E-06	0.0189	ACATTAAATAT
	Zpz_sc00235.1.g00210.1.sm.mkhc	HD-ZIP	1.06E-05	0.019	AAAATAATTGT
	Zpz_sc00158.1.g00400.1.sm.mk	Dof	1.11E-05	0.0261	CAAAAAGTAAT
	Zpz_sc04854.1.g00020.1.sm.mkhc	GATA	1.11E-05	0.033	GATATAGATCTAT
	Zpz_sc00187.1.g00470.1.am.mkhc	CPP	5.38E-05	0.0361	TATTTAAATT
	Zpz_sc00443.1.g00380.1.sm.mk	YABBY	3.50E-05	0.0396	TATGATAA
	Zpz_sc01162.1.g00080.1.sm.mkhc	MYB_related	1.75E-05	0.0417	CAGATATTT
	Zpz_sc00403.1.g00170.1.sm.mk	WRKY	1.45E-05	0.0492	CGGTCAAC

ERF: Ethylene responding factor; *Zj*: *Z. japonica*; Zm: *Z. matrella*; Zp: *Z. pacifica*.