

Computational study of environmental stress-related transcription factor binding sites in the promoter regions of maize auxin response factor (ARF) gene family

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Abstract

Auxin response factors (*ARF*) gene family plays key roles in plant development and act as transcription factors (TFs) in the regulation of gene expression. An extensive bioinformatics analysis including analysis of conserved motifs, chromosomal map, phylogenetic relationships, and expression profiles were performed for the maize *ARF* gene family. In this study, a set of publicly available 38 *ARF* maize (*Zea mays*) nucleotide sequences were downloaded. Using microarray data, a bioinformatics search for identification of TFs in *ARF* genes using plant promoter analysis (PlantPAN) was carried out. The 38 maize *ARF* genes were categorized into three groups (Class I, II, and III). *ARF* genes have been studied by molecular methods in several different plant species however to better understand the mechanisms of these genes more studies are needed. Gene cluster analysis showed that the same set of genes on the chromosomes were positively correlated with the same number of gene clusters. Several TFs including AP2/ERF, ERF, WRKY, bZIP, bHLH, GATA, and NAC were identified in the promoter regions. These TFs are responsible for modulation of several biotic stress-responsive genes. The main aims of the present study were to obtain genomic information for the *ZmARF* gene family and their expression under abiotic and biotic stresses.

Keywords: expression study; heatmap; transcriptional factor binding site; *zmARF*; *Zea mays*

Introduction

Auxin, a plant hormone involved in various stages of plants growth and development, is involved in apical dominance, tropisms, and vascular patterning. Auxin plays a key role in cell division and cell expansion during the developmental stages and in regulation of a variety of physiological processes including lateral root initiation and shoot elongation (Di *et al.*, 2016). During the last decade, many studies have reported on the rapid effects of auxin on gene expression and regulation. It has been shown that auxin is involved in regulation of a large number of genes having roles in growth and developmental processes in *Arabidopsis* and other plant species (Guilfoyle and Hagen, 2012; Di *et al.*, 2016). According to many reports, these processes are controlled by genes responsive to auxin at the molecular level.

Auxin response factors (*ARF*) are TFs that regulate auxin responsive genes. So far, members of *ARF* cascades from *Arabidopsis*, rice, tomato and maize have been identified (Wang *et al.*, 2007). *ARFs* are involved

in diverse biological and physiological processes and are essential for their potential applications for the development of improved stress-tolerant transgenic crop plants. *ARF*s genes can act as either an activator or a repressor of the transcription of auxin-responsive genes (Saidi *et al.*, 2020). *ARF* regulates early responsive genes such as Aux/IAA, GH3, and the smallest auxin uptake (SAUR) gene family, activating or inhibiting these genes by binding to auxin-responsive elements (AuxREs). AuxREs elements may include the following sequences of TGTCTC, TGTCAC and/or TGTCCC elements in their promoter region (Hagen and Guilfoyle, 2002). Auxin transcription factors are considered as significant gene family in response to auxin (Wang *et al.*, 2015). The first step in identifying and studying gene function is the use of data from databases using bioinformatics tools. The main aim of bioinformatics is to increase the understanding of biological processes, co-expressed gene network, and prediction of TFBs (Hogeweg, 2011).

The presence of TFBs in many of the studied genes causes the plant to respond appropriately to abiotic and biotic stresses such as physiological processes and plant growth and reproduction (Greenham and McClung, 2015). The promoter analysis of auxin-responsive genes makes identification of TFBs or AuxREs possible (Hagen and Guilfoyle, 2002). Recently, this gene family has extensively been investigated using molecular and bioinformatics analysis. Molecular analyses of ARF genes on several crops such as citrus, *Medicago truncatula*, and *Gossypium raimondii* have been performed (Li *et al.*, 2015; Sun *et al.*, 2015).

Most ARF proteins are made of three domains namely N-terminal DNA-binding domain (DBD), the variable middle region that functions as an activation domain (AD) or repression domain (RD), and a carboxy-terminal dimerization domain (CTD) which is involved in protein-protein interactions by dimerizing with auxin/indole-3-acetic acid (Aux/IAA) gene family (Greenham and McClung, 2015). In some studies of *A. thaliana*, it has been indicated that glutamine (Q)-rich middle regions act as activation regions but that serine (S)-rich, serine and proline (SP)-rich regions function as repression regions in ARFs (Tiware *et al.*, 2003).

Several *ARF*s also play an essential role in reproductive development. Recently, functional studies have demonstrated that *ARF* genes play key role in signal transduction during plant organ development. In *Arabidopsis thaliana*, ARF2 expresses leaf senescence (Lim *et al.*, 2010), root formation and flower organ senescence (Ren *et al.*, 2017).

Arabidopsis ARF10 and ARF16 have essential roles in the process of root development, and the auxin signals cannot bypass them to initiate cell production (Wang *et al.*, 2005). Furthermore, ARF7 and ARF19 may regulate *Arabidopsis* lateral root (LR) formation initiation by interacting with IAA14 (Ito *et al.*, 2016).

Several ARFs also play an essential role in reproductive development. In *Arabidopsis*, ARF6 and ARF8 promote jasmonic acid production and flower maturation (Nagpal *et al.*, 2005) and influence the developing floral organs (Tabata *et al.*, 2010).

In this study, we investigated the maize *ARF* gene number, chromosomal map, and promoter analysis. The phylogenetic analysis of *ARF* genes would be useful for studying and understanding of their roles in plants. The study of this gene family would be a useful strategy for increasing tolerance of crop plants against environmental stresses. The main aims of this study were to better understand the *ARF* gene family and their relationships with Aux/IAA gene families, to obtain genomic information for the *ZmARF* gene family, and their expression under abiotic and biotic stresses.

Materials and Methods

Identification of maize ARF gene family, phylogenetic analysis of ARF genes, and conserved motifs

Maize ARF nucleotide sequences were downloaded from (<http://grassius.org>). Alignment of the sequences of the *ARF* genes was performed using CLUSTALW program in MEGA software version 6 (Tamura *et al.*, 2013). Phylogenetic tree was constructed using the Neighbor-Joining (NJ) method and the diagrams of phylogenetic trees were drawn with MEGA6 (<http://www.megasoftware.net/mega.php>) (Tamura *et al.*, 2013). In addition, confirmation of phylogeny tree was done using the bootstrap method with 1000

replicates. The *Z. mays* ARF protein sequences were downloaded from (www.grassius.org). ExPASy server (http://web.expasy.org/compute_pi/) was employed to predict the theoretical isoelectric point (pI) and the molecular weight (Mw) of each ZmARF protein. ZmARF transcription factors were identified using MEME (Suite version 4.9.1; <http://meme-suite.org/tools/meme>) according to the following criteria: maximum number of 15 motifs and an optimum width of 8-50 amino acids (Table 1).

Table 1. Characteristics of ZMARF gene family in maize

Protein Name	Gene Locus	Synonym/Gene Name	Length(aa)	molecular weight	isoelectric point
ZmARF1	GRMZM2G169820	ARFTF1	1086	120169/26	5/89
ZmARF2	GRMZM2G153233	ARFTF2	681	74543/03	8/08
ZmARF3	GRMZM2G078274	ARFTF3	817	90993/05	5/95
ZmARF4	GRMZM2G034840	ARFTF4	936	102930/48	5/78
ZmARF5	GRMZM5G808366	ARFTF5	513	55449/89	5/97
ZmARF6	GRMZM2G122614	ARFTF6	273	29733/73	7/06
ZmARF7	GRMZM2G475263	ARFTF7	686	76716/48	5/92
ZmARF8	GRMZM2G352159	ARFTF8	750	83276/87	6/28
ZmARF9	GRMZM2G073750	ARFTF9	888	97844/57	5/72
ZmARF10	GRMZM2G338259	ARFTF10	805	89801/59	5/9
ZmARF11	GRMZM2G056120	ARFTF11	688	74991/77	6/89
ZmARF12	GRMZM2G437460	ARFTF12	708	77967/76	6/67
ZmARF13	GRMZM2G378580	ARFTF13	850	93150/67	6/56
ZmARF14	GRMZM2G137413	ARFTF14	511	56730/84	6/26
ZmARF15	GRMZM2G081406	ARFTF15	689	73684/98	6/91
ZmARF16	GRMZM2G028980	ARFTF16	914	100942/68	5/7
ZmARF17	GRMZM2G159399	ARFTF17	644	70964/01	6/52
ZmARF18	GRMZM2G035405	ARFTF18	729	81995/32	6/5
ZmARF19	AC207656.3_FG002	ARFTF19	716	77519/86	6/67
ZmARF20	GRMZM2G102845	ARFTF20	1147	127239/65	5/79
ZmARF21	GRMZM2G390641	ARFTF21	700	75511/41	7/16
ZmARF22	GRMZM2G089640	ARFTF22	631	69924/03	5/87
ZmARF23	GRMZM2G441325	ARFTF23	680	73940/89	6/4
ZmARF24	GRMZM2G030710	ARFTF24	736	80418/42	7/87
ZmARF25	GRMZM2G116557	ARFTF25	806	90231/11	6/09
ZmARF26	GRMZM5G874163	ARFTF26	683	74119/81	6/68
ZmARF27	GRMZM2G160005	ARFTF27	1053	116746/55	6/2
ZmARF28	GRMZM2G006042	ARFTF28	813	89705/5	6/81
ZmARF29	GRMZM2G086949	ARFTF29	958	105292/29	6/03
ZmARF30	GRMZM2G475882	ARFTF30	834	92500/92	6/46
ZmARF31	GRMZM2G023813	ARFTF31	462	50564/12	5/38
ZmARF32	GRMZM2G181254	ARFTF32	418	46826/12	6/57
ZmARF33	GRMZM2G179121	ARFTF33	446	48728/8	8/48
ZmARF34	GRMZM2G081158	ARFTF34	897	98369/19	5/84
ZmARF35	GRMZM2G317900	ARFTF35	975	107537/87	6/17
ZmARF36	GRMZM2G702026	ARFTF36	677	75129/44	5/93
ZmARF37	GRMZM2G075715	ARFTF37	496	56934/86	9/27
ZmARF38	GRMZM2G005284	ARFTF38	588	63905/47	8/88

Expression study of ARF genes

In order to determine the differential gene expression under different stresses, *ARF* gene expression data was extracted by Genevestigator from *Zea mays* database using mRNA-Seq Gene Level *Zea mays* (ref: AGPv4) (ZM_mRNASeq_MAIZE_GL-0) and 'Perturbations' tool. The fold change in gene expression was detected using filter 2 folds as benchmark. The fold changes in the expression of *ARF* genes under different environmental stresses were used to generate gene expression heatmap using Genevestigator (<https://genevestigator.com/gv/>) with Red/Green colour scheme as markers where "Red" and "Green" colors represent up and down-regulation of respective genes. Genevestigator (<https://genevestigator.com/gv/>) was performed to determine the expression of *ARF* genes in different developmental stages in maize.

Mapping ZmARFs genes on maize chromosomes and analysis of transcriptional factors binding sites

A total of 38 *ARF* genes were mapped to chromosomes 1-10. In order to show chromosomal locations of *Zea mays ARF* genes, the MapChart software version 2.3 (Voorrips, 2002) was downloaded from <https://www.wageningenur.nl/en/show/Mapchart-2.30.html>. A total of 1.5 kb upstream sequence was analyzed for each *ARF* gene by PlantPAN 3.0 software (<http://plantpan3.itps.ncku.edu.tw/>) for detection of TFs in promoter regions on both strands (Blanco *et al.*, 2006). PlantPAN is an appropriate resource for estimating regulatory elements and reconstructing transcriptional regulatory networks of plant genes. The database released 17230 TFs which were collected from 78 plant species.

Results and Discussion

ARF transcription factors play key roles in various plant growth and development. In the present study, we surveyed 38 *ZmARF* genes in different stress condition and developmental stages.

Identification of ZmARFs and phylogenetic analysis of ARF genes

The predicted proteins encoded by *ZmARF* genes varied from 418 (*ARF13*) to 1147 amino acids (*ARF20*), with corresponding molecular weights from 46.84 kDa (*ARF13*) to 127.24 (*ARF20*) kDa, and the theoretical isoelectric points ranging from 5.38 (*ARF33*) to 9.27 (*ARF13*). The phylogenetic tree of all maize *ARF* nucleotide sequences was generated using MAGE v7.0 program by the N-J method. All *ZmARFs* genes were classified into three major classes -I, II, and III (Figure 1). Classes I, II, and III each contained 22, 2, and 13 members, respectively. These results are similar to the grouping of these genes in related studies (Li *et al.*, 2015; Saidi and Hajibarat, 2018).

Chromosomal locations of ZmARFs gene

The distribution of 36 members of *ZmARF* family distributed on 10 maize chromosomes is shown in Figure 2. Five *ZmARFs* genes were located on each of the chromosomes 1, 5, and 10; four genes on chromosomes 1 and 6; seven genes on chromosome 3, and, only one gene on chromosome 9 (Figure 2). Due to the location of these genes on maize chromosomes, all chromosomes contained *ARF* encoding sequences except chromosome 7. In this study, we found gene duplication in chromosomes 4, 5, and 10. Although, genes were distributed on 10 chromosomes but only one gene cluster was found on each of the chromosomes 1, 2, and 10 (Figure 2). The clustering patterns of these sequences were similar with their placement on each chromosome. Our results were in agreement with those reported by other researchers (Liu *et al.*, 2012; Saidi and Hajibarat, 2019).

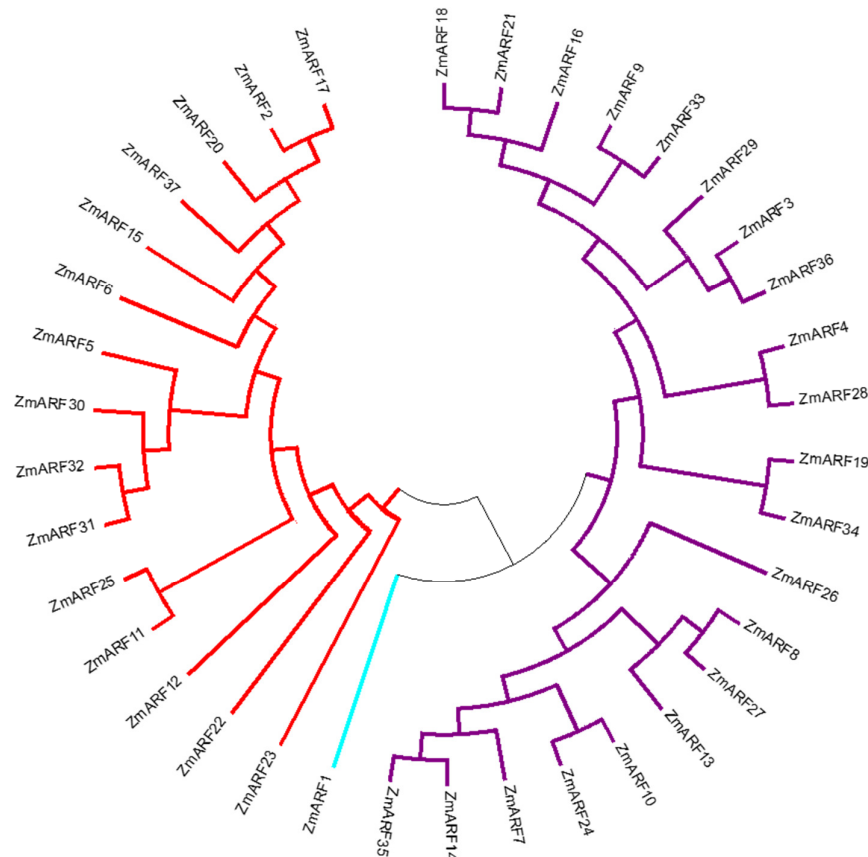


Figure 1. Phylogenetic tree of maize *ARF* genes. The unrooted tree was generated using the MEGA v7.0 program with the Neighbor-Joining method. All thirty-eight maize ARFs were classified into three groups

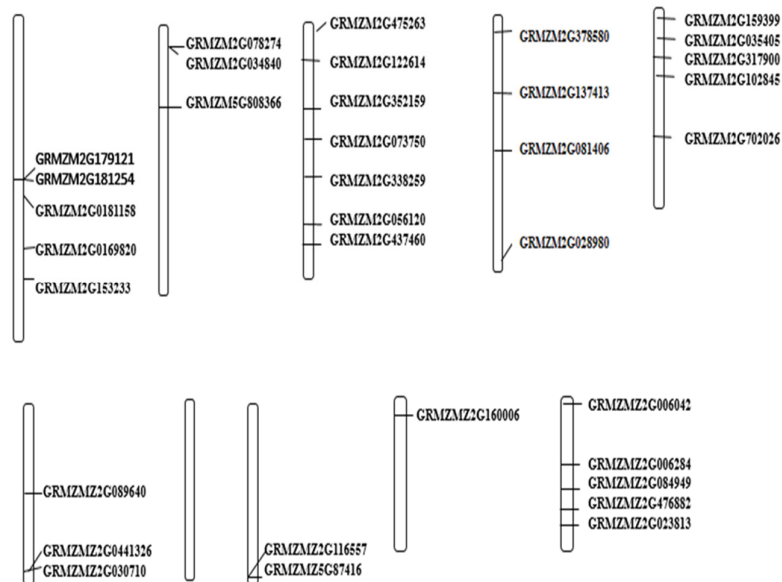


Figure 2. Chromosomal distribution and expansion patterns of maize *ARF* genes

Conserved motifs

Most ARFs consist of three domains such as DNA-binding domain (DBD), a middle region that functions as an activation domain (AD) or repression domain (RD), and a C-terminal PB1 protein-protein interaction domain (PB1) (Li *et al.*, 2015). The similar distribution of motifs in the ARF proteins suggests an evolutionary relationship among them. The sites of different motifs on the protein sequence may help to determine the differences in the performance of genes under different stress conditions (Liao *et al.*, 2017). To identify the conserved motifs in this gene family, the protein sequences of *ZmARF* genes were surveyed by MEME software (Figure 3).

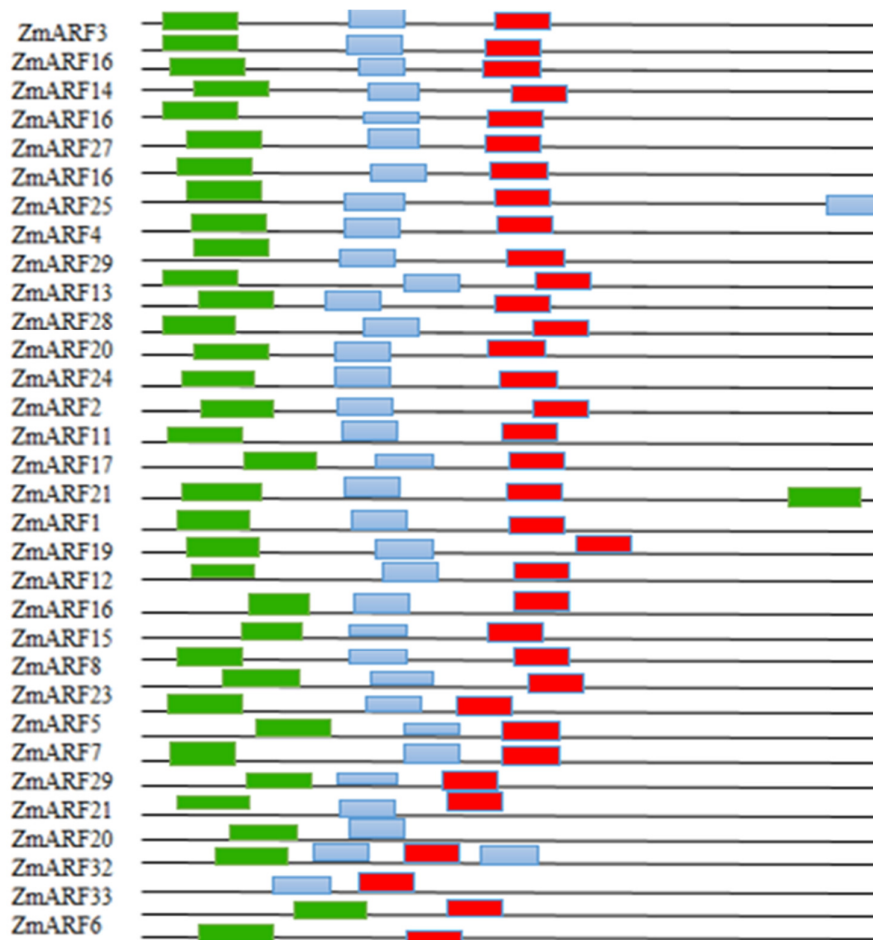


Figure 3. Conserved *ZmARF* protein motifs in maize, recognized by MEME database. Motifs 1-3 are identified by different colors

ZmARF gene expression study

Expression profiles of *ZmARF*s genes for *Colletotrichum graminicola*, *Fusarium verticillioides*, heat, and submergence were retrieved. The expression profiles of *ZmARF* genes in response to different abiotic stresses (submergence, heat) were also analysed. Up-regulation was observed in *ZmARF6* under abiotic and biotic stresses; *ZmARF1*, *ZmARF7*, *ZmARF18* under biotic stresses such as (*C. graminicola* and *F. verticillioides*); and in *ZmARF6* under heat stress. Down-regulation was observed in *ZmARF16* under submergence; *ZmARF11* under *C. graminicola*, *F. verticillioides*, submergence, and heat stress. When tomato is encountered with biotic stresses, *SLARF1*, *SLARF4*, *SLARF6*, *SLARF10* and *SLARF18* genes were activated

with a significant increase in expression, however some genes such as *SLARF3* and *SLARF5* were significantly repressed (Figure 4a).

Tomato *ARF* genes were induced in response to flagellin in only six hours. Therefore, the expression of almost all *SLARF* gene family is repressed except for *SLARF6* and *SLARF18* which showed a twofold expression. Our findings revealed that *ZmARF6* and *ZmARF18* genes increased expression significantly in response to biotic stresses, in contrast to the results reported by (Bouzroud *et al.*, 2018). Expression analysis revealed that *ZmARF* was down-regulated in response to *Colletotrichum graminicola*. A recent report has shown that *ZmARF4* was down-regulated in response to *C. graminicola* and activated in response to auxin (de Jesus Miranda *et al.*, 2019).



Figure 4a. Heatmap analysis of *ZmARF* genes in response to environmental stresses
The microarray data for expression of 16 *ZmARF* genes under various abiotic and biotic stress conditions

Microarray data indicated an up regulation of all expressed *ZmARF* genes during different developmental stages. *ZmARFs* (1-38) are main constituents of biological processes and have different functions in plant species. Our finding revealed that *ZmARF1* is highly expressed in all developmental stages; this may be due to the presence of different TFBs (Figure 4b). As a result, these genes can play key roles in different growth and developmental stages and under abiotic and biotic stresses. *ZmARF5*, *ZmARF32*, and *ZmARF17* genes were down-regulated during several developmental stages such as dough stage, fruit formation, and seedling stage. *ARF8* is reported to regulate fertilization and fruit development (Goetz. *et al.*, 2006) and *ARF6* and *ARF8* are essential players in flower maturation and flowering processes (Finet *et al.*, 2010). Our analysis showed that *ZmARF6* and *ZmARF8* are involved in flower and fruit formation in maize which is in line with the findings reported by others (Goetz *et al.*, 2007; Finet *et al.*, 2010).

ARF6 and *ARF8* genes regulate development at reproductive and vegetative stages with overlapping functions (Goetz *et al.*, 2007). *ARF19* acts redundantly in controlling leaf expansion and lateral root growth (Wang *et al.*, 2007). Our findings revealed that *ARF2* was up-regulated in all different developmental stages (Figure 4b). A study showed that *ARF2* control leaf senescence and floral organ abscission in *Arabidopsis* (Ellis *et al.*, 2005). *ARF3* gene plays a key role in the formation of meristem, while *ARF4* plays an important role in organ polarization (Hunter *et al.*, 2006). The presence of *ARF5* is essential for the formation of flower and root embryos whereas, *ARF7* gene expression was increased during all developmental stages (Figure 4b). However, a report indicated that *ARF7* acts as a negative regulator of fruit formation (ex., pollination and fertilization), as well as an intermediary between gibberellins and auxin messaging during tomato fruit formation (Wilmoth *et al.*, 2005). *ARF4* is involved in controlling sugar metabolism during tomato fruit development (Zouine *et al.*, 2014).

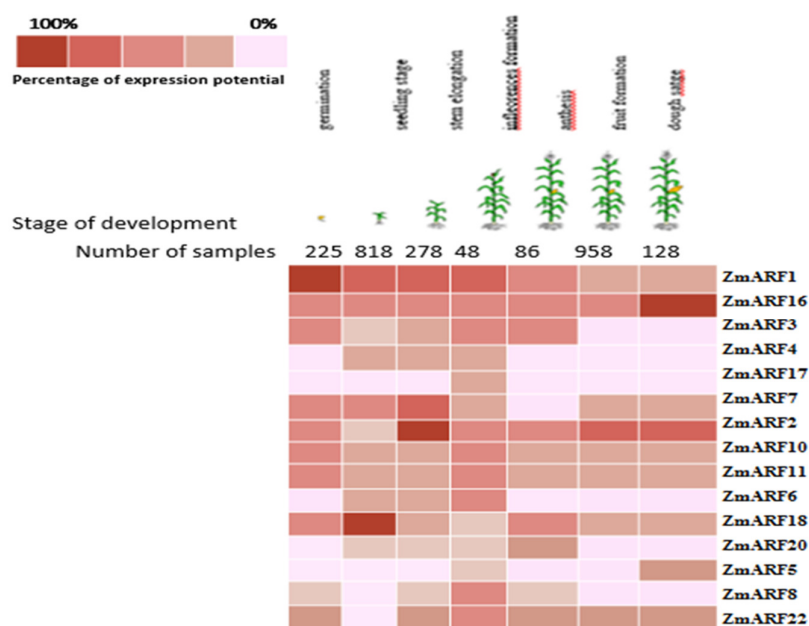


Figure 4b. Heatmap analysis of expression of *ZmARF* genes at different developmental stages of *Z. mays*

Analysis of transcriptional factors binding sites

AP2; ERF participates in response to auxin, cytokinin, abscisic acid, and jasmonate signals. In rice, an AP2; ERF OsCRL5 is induced by exogenous auxin, treatment which in turn inhibits cytokinin signal transduction by enhancing the activities of two repressors (Khurana *et al.*, 1988). Co-expression of a gene(s) with a transcription factor gene(s) can provide clues about regulatory mechanisms (Hirai *et al.*, 2007).

Among the six TFs surveyed in this study, the presence of AP2/ERFs was the highest in the promoters of *ZmARF* gene families of maize. Analysis of TFs in the promoter sequences provide a good understanding of plant responses in stress conditions. Genes promoter analysis revealed that AP2; ERF, ERF, bZIP, GATA, and WRKYs were present in majority of the promoter regions of auxin-responsive genes.

There are TGTC core element in AP2; ERF (GTGTCGGTTT), ERF (GTGTCGGTTT), bZIP (TGAGCTGTCT), WRKY (TGTCACA), and C2H2 (CAGTGTC) TFs sequences. TGTC core can bind in promoter regions with TFs and provide to create resistance in response to environmental stresses. Among 38 *ZmARF* gene promoters, the highest number of bZIPs was observed in *ZmARF11-12* promoter.

AP2/ERFs are reported to be involved in improving resistance to biotic stresses. In addition, few of the AP2/ERF TFs have been reported to enhance resistance to biotic and abiotic stresses, simultaneously (de Jesus Miranda *et al.*, 2017). According to other reports many of the AP2/ERFs in the upstream genes can enhance resistance to biotic and abiotic stresses (Kasuga *et al.*, 1999; Berrocal-Lobo *et al.*, 2002). For example, the bZIP TF is reportedly involved in the regulation of abiotic and biotic stresses. The role of bZIP proteins in response to biotic stress is widely known (da Silva and de Oliveira, 2014).

Several studies have validated the role of bZIP in ABA signalling in rice. Also, they have been found to play a regulatory role upon drought stress during vegetative stages (Fujita *et al.*, 2013). bZIP transcription factors are a gene family which regulates several steps of plant's life such as light signalling, pathogen defence, flower development, and abiotic stress signalling (Rahaie *et al.*, 2011). Additionally, *ZmARF16* gene did not contain any WRKYs in its promoter region. WRKYs are of particular interest as they are involved in diverse environmental stress responses as well as in physiological/biological processes (Jiang *et al.*, 2015).

Moreover, *OsWRKY* regulates cascade of signalling pathways mediated by jasmonic acid and salicylic acid for protecting plants from bacterial and fungal infections (Chu *et al.*, 2015). In addition to the biotic stress involvement, a number of WRKY family members are also associated with abiotic stress responses in rice (Zheng *et al.*, 2006). The up-regulation of some members of WRKY TFs have been found to implicate salt, drought, and heat tolerance in plants (Yang *et al.*, 2017).

All AP2/ERFbs were found to be distributed throughout in *ZmARF* promoter regions. The presence of AP2; ERFbs in promoter regions can play a role in environmental and hormonal stresses. In the present study, the largest number of TFbs (four TFbs) were detected in *ZmARF*10-12 followed by *ZmARF*35.

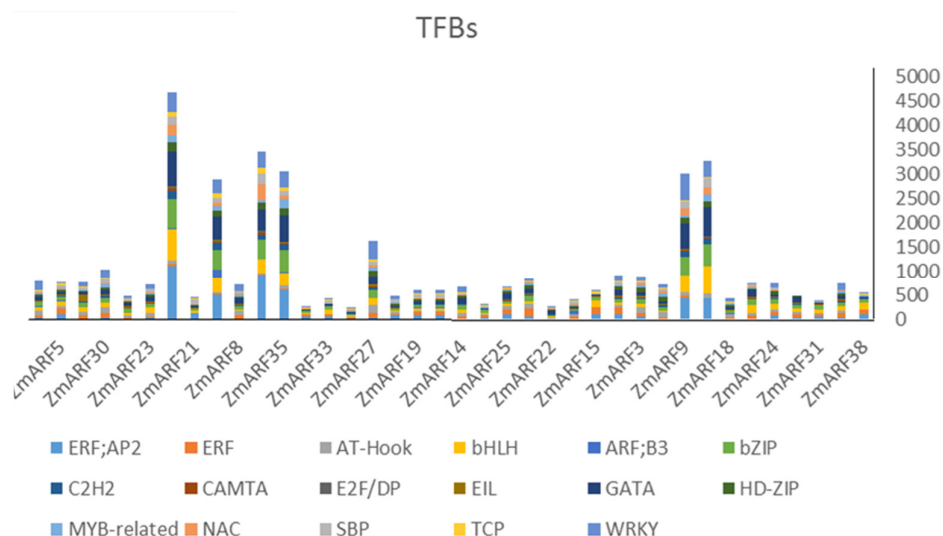


Figure 5. Transcription factor binding sites detected in the upstream of promoter regions and their frequencies in each gene

Conclusions

In the present study, an extensive analysis of maize *ZmARF* genes was conducted to evaluate their chromosomal distribution, phylogenetic tree, conserved motifs, transcriptional factor binding sites (TFBs) in promoter regions, and expression. The number of ARF family members varies in different plants which can be due to differences in their genomic size. The chromosomal distribution and phylogenetic relationships of the *ARF* genes provide a valuable tool into the evolutionary status of maize. However, there are few studies of *ARF* genes in maize. In this study, we used maize *ARF* gene sequences and applied various bioinformatics tools to gain more knowledge about biological/physiology processes. Analysis of 38 *ZmARF* promoter regions revealed the presence of different types and frequencies of TFBs among genes. In our analysis, several TFBs including AP2/ERFbs, WRKYbs, bZIPbs, bHLHbs, GATAbs, and ERFbs were identified in the promoter regions of *ZmARF* genes. Gene cluster analysis showed that the same set of genes on the chromosomes were positively correlated with the same number of gene clusters. Further analysis is required to confirm these *ZmARF* promoters for future utilization in crop breeding as stress inducible promoter.

Authors' Contributions

Both authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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