

Identification and Distribution of Anaerobic Responsive Elements (AREs) in Genes Functional Categorization of *Arabidopsis thaliana*

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Abstract

The evolution of biota had been tightly linked with formation of atmospheric di-oxygen (O₂). Since the shift from anaerobic to aerobic life results in reprogramming gene expression through the regulatory interactions between transcription factors and their target sequences, the anaerobic responsive elements (AREs). The purpose of this study is to consider AREs; the most frequent and divergent motif in comparison to eighty four plant cis-regulatory elements, in order to clarify their biological function in *Arabidopsis thaliana*. A genome wide survey revealed the frequency, occurrence number and positional state of each anaerobic element in *Arabidopsis* genome. The evidence was obtained that among all AREs, TTCCCTGTT, (A/C/G)AAAAACAAA and AGCAGC *in silico* discovered elements, are noteworthy responsive elements in *Arabidopsis*. In addition to, our data suggest that the evolutionary process of anaerobic responsive elements may have been directed toward gradual decrease in occurrence numbers of AREs and gradual increase of their ineffectiveness in *Arabidopsis* genome. We also classified *Arabidopsis* genes according to existence of ARE elements in promoter regions of them to seven main groups including expression, primary metabolism, secondary metabolism, signaling, growth and development, stress and misc.

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Submission Date: 10/15/2014

Accepted Date: 12/23/2014

Keywords: Anaerobic Response Element, Cis-Regulatory Element, Gene Expression, Positional Significant Element

Introduction

The early atmosphere of earth had been empty of oxygen that formation and increasing atmospheric di-oxygen provided the aerobic conditions for organisms [1]. In 700 million years ago, the first terrestrial plants were probably under anaerobic condition and symbiotic relationships, for example; first photosynthetic organisms (green algae) with some fungi lineage increased their ability to survive and reproduce [2]. It seems that first plant colonization of land had confronted with harsh condition, because they had to change their life of style from anaerobic to aerobic. The anaerobic growth media generate stress condition for plants and they have a major effect on gene expression and protein synthesis [3, 4].

The anaerobic energy metabolism in plant adopts to produce the maximum of ATP production through the fermentation pathway [5], that specific interaction between anaerobic responsive elements (AREs) and transcription factors (TFs) can regulate the transcriptional levels of genes involved in ATP production pathways. Previous researches indicate that nineteen AREs with different sequences are responsible for regulation of genes in *Arabidopsis* and *Maize* [6-9].

On the other hand, AREs indicated sequence variation and number, but only three of them were experimentally verified and results showed that one protein of MYB family (AtMYB2 transcription factor) binds to TGGTTT ARE element [10, 11]. The most important feature of ARE elements seems to be the frequencies in one or more

sequences of ARE motifs in promoter regions of plant genes [12]. Additionally, the distance from transcription start site (TSS), orientation, order, and spacing between elements are key factors in the concentration of active nuclear transcription factors (TFs) and transcriptional regulation of responsive genes [13]. Unfortunately, smallness, non-conservation and phenomena such as mutations, insertions or deletions during the evolution of cis-regulatory sequences caused the major challenge to identify co-expression or co-regulated genes [12, 14, 15]. In the recent years, wide variety of database including BAR [16], PLACE [17], Plant CARE [18] and TRANSFAC [19], have been developed and focused on compiling cis-regulatory elements and element containing genes. In this study, we found that ARE motifs were the most frequent and divergent rather than other cis-regulatory elements involved in promoter regions of genes in *Arabidopsis*.

It can discover the interesting facts about the evolutionary behavior of AREs in *Arabidopsis* genome. Our analysis demonstrated that three elements are the most probable responsive elements among all *in silico* reported AREs and there are evidences to corroborate the common biological function of AREs in abiotic stress. We also categorized *Arabidopsis* genes according to existence of ARE elements in promoter regions of them to seven main groups including expression, primary metabolism, secondary metabolism, signaling, growth and development, stress and misc.



Material and Methods

Identification of genes according to common cis-regulatory element in promoter region

All candidate cis-regulatory elements involved in plant genome were gained by PlantCARE database [18] and used to find genes with common element in promoter regions of *Arabidopsis* genes. The analysis was performed by Promomer tools of BAR database; a web software which presents number of element containing genes, offset of elements position and occurrence numbers (hit numbers) of each element in promoter sequences of *Arabidopsis* genes [16].

Identification of genes based on different AREs

All ARE elements (19 elements) involved in anaerobic responsiveness were determined by using literature survey [6, 7, 8, 9]. Similar to other elements, Promomer tools [16] were applied to find genes contained the same ARE sequence. Then, Pearson correlation coefficients (PCC) were calculated between hit numbers of AREs and elements length. This score was also measured between hit numbers of AREs and elements GC content. The PCC for all AREs were calculated by SPSS 17.0 software.

Positional analysis of AREs in promoter region

Nineteen gene classes were formed based on each ARE motifs which consisted of 90 randomly selected genes. These classes were utilized to calculate positional occurrence frequency of each ARE motif. Promoter sequences of genes (a 1000 bp upstream region from transcription starting site) were divided into five 200 bp regions and scored one or zero for existence and non-existence of analyzed motif in every region, respectively. Then, nineteen matrixes with 90 rows and 5 columns were created and the frequency analysis carried out according to the below equation for each AREs:

$$A_j = \frac{\sum_{i=1}^{n=90} f_{ij}}{\sum_{i=1}^{n=90} \sum_{j=1}^{m=5} f_{ij}} \times 100$$

A_j denotes the percentage of element l occurrence for j= 1, ..., 5

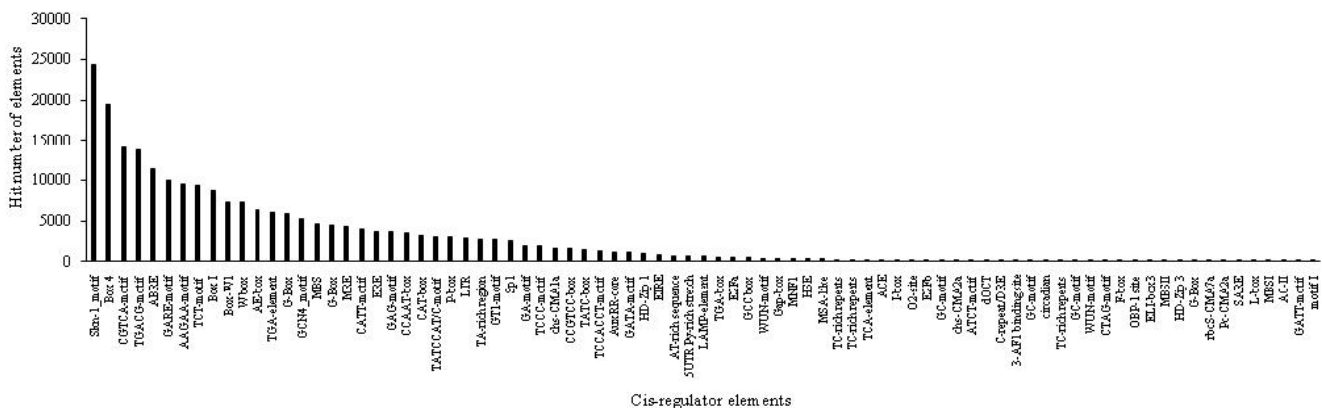


Figure 1. The hit numbers of 84 cis-regulatory elements in promoter region of *Arabidopsis* genes. The results were obtained by using elements sequences in the promomer tool of BAR data base. For motifs with different sequence, the hit number was calculated by summation of hit numbers.

i₁= gene₁, element₁, i₉₀= gene₉₀, element₁
j₁=elementfrequency_{-1000,-800}, ..., j₅= element frequency_{-200,-1}

Finally, PCC score was computed between percentages of AREs elements and five numeric distances from transcription start site (-200,-400,-600,-800 and -1000 respectively). Motifs with < 0.05 selected as significant positional elements.

Functional classification of genes based on possessing AREs

At the first, genes containing of each ARE motifs were separated based on hit number and then each subclass (or hit based classes) of genes was used for functional classification. The classification was performed through Classification SuperViewer Tool of BAR database. In addition, MAPMAN method was selected as classification source [20]. In general, seven functional classes including the expression, Secondary metabolism, primary metabolism, signaling and transport, growth and development, stress and misc were created.

Results

Genome-wide analysis of Arabidopsis thaliana based on cis-regulatory elements

Hit number of 84 cis-regulatory elements was comparatively analyzed by using promoter in the 1000 bp upstream regions of *Arabidopsis* genome. Apart from anaerobic responsive elements (AREs) as shown in Figure 1, the Skn-1-motif as the first most abundant element (24,351 hit numbers) founded by 47.4% in *Arabidopsis* genes and GATT and I motifs (both of them had only two hit) had less frequency than other elements.

Literatures revealed that nineteen AREs were experimentally and putatively introduced until now [6-9]. Genome-wide assessments of *Arabidopsis* demonstrated that the AA(A/G)ATT element by 82.89% occurrence (78822 hit number) as the highest frequency and the TTCCCTGTT element with only 0.241% (79 hit numbers) as the lowest frequency founded in promoter regions of 33602 genes of *Arabidopsis* (TAIR database).

These results illustrated that AREs were the most widespread cis-regulatory elements in *Arabidopsis* genome in comparison to other elements (Fig. 2). In addition, statistical assessments by calculating the PCC showed that the hit number of AREs had not significant correlation with motifs length and GC content (-0.46, -0.32 respectively), therefore, distribution of AREs in *Arabidopsis* genome were independent of their length and GC content.

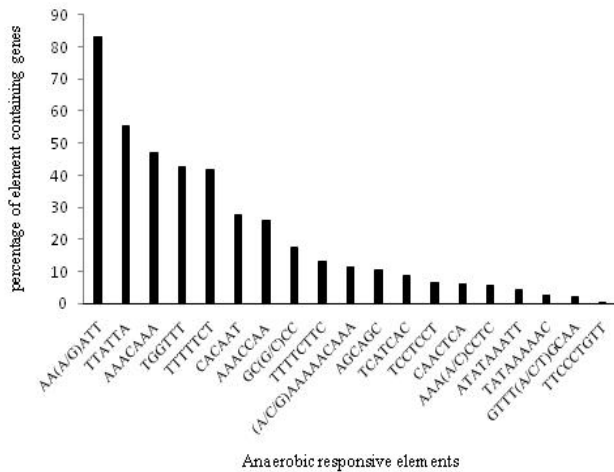


Figure 2. The percentage of anaerobic responsive elements (AREs) containing genes in *Arabidopsis thaliana* genome. The results were calculated of dividing the genes which had each one of AREs in their promoters on 33602 genes of *Arabidopsis thaliana*. Among all AREs, the AAA(A/G)ATT elements were the most frequent element in *Arabidopsis* genome.

Positional distribution of AREs

All *Arabidopsis* genes were classified into nineteen groups based on each AREs. Every group consisted of genes with a typical ARE and also some genes that were fairly common between gene classes. Ninety random selected genes from each class were analyzed to determine whether AREs occurrence have any tendency toward a specific region of promoter or not. The percentages of all AREs frequencies were calculated in five regions of promoter (1000 bp upstream region were divided into five 200 bp) and then PCC computed between percentage of element frequency and distance from TSS. Results indicated that among all AREs, the TTTTCTTC (PCC= 0.932, sig.0.02), (A/C/G)AAAAACAAA (PCC= 0.915, sig. 0.03) and AGCAGC (PCC= -0.949, sig. 0.014) motifs were positional significant elements (PSE) and had a tendency towards specific regions of promoter (Fig. 3). The TTTTCTTC and (A/C/G)AAAAACAAA motifs were preferably located near TSS, whereas AGCAGC motif occupied a region of promoters with a long distance from TSS (Fig. 3). However, positional analysis of other AREs showed no preferable occurrence in particular region of promoters.

Functional classification of AREs related genes

To address the biological function of AREs containing genes, the SuperViewer of Bar database were used as the

gene functional categorization tool in cellular processes [21, 22]. For each ARE, all genes with the same hit number were separately classified. Apart from stress and misc classes, program outputs were rebuilt based on the highest functional similarity and created highlight functional categories.

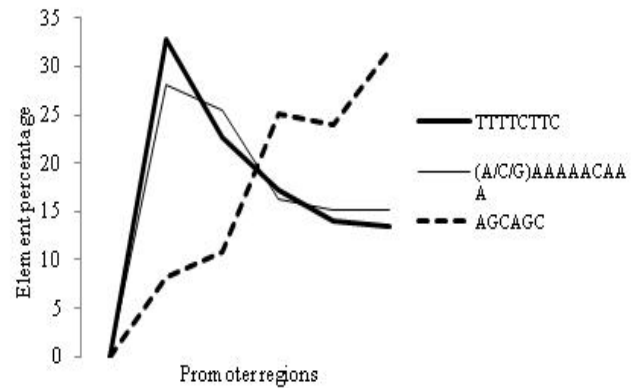


Figure 3. The positional significant elements (PSE).The X axis indicates that 1000 bp upstream region were divided into five 200 bp (-200, -400, -600, -800 and 1000, respectively). The percentage of TTTTCTTC and (A/C/G)AAAAACAAA elements has a bias near TSS whereas occurrence percent of AGCAGC was significantly in region with a long distance from TSS.

Finally, seven main groups including expression, primary metabolism, secondary metabolism, signaling, growth and development, stress and misc classes were created for all ARE elements (Fig. 4).

The expression class was consisted of protein, RNA and DNA structural and synthesizing genes, the secondary metabolism group was composed of hormone, lipid, amino acid, nucleotide, major and minor carbohydrates, polyamines, and nitrate metabolizing genes and also sulfur assimilation, metal handling and biodegradation of xenobiotic involving genes. The primary metabolism was included of photosynthesis, mitochondrial electron transport/ATP synthesis, glycolysis, C1-metabolism, gluconeogenesis/glyoxylate cycle and fermentation involving genes. Finally, the signaling and transport group was made of signaling, transport, redox and TCA/org transformation genes and the growth and development was consisted of cell cycle, cell division, development and cell wall structural involving genes. Program outputs merged in five groups along with stress and misc groups of program itself. Our data also demonstrated that the members of genes assigned with expression and signaling and transport classes were predominately more than others functional groups. The number of genes involved in secondary metabolism and growth and development had a little more two kind of ARE elements including TCCTCCT and GTTT(A/C/T)GCAA elements than the signaling and transport involving genes. As it is showed in Figure 4, the majority of genes contained one ARE and number of genes in seven functional classes decreased by increasing in hit number, although, this declining pattern did not happen in same manner for all AREs.

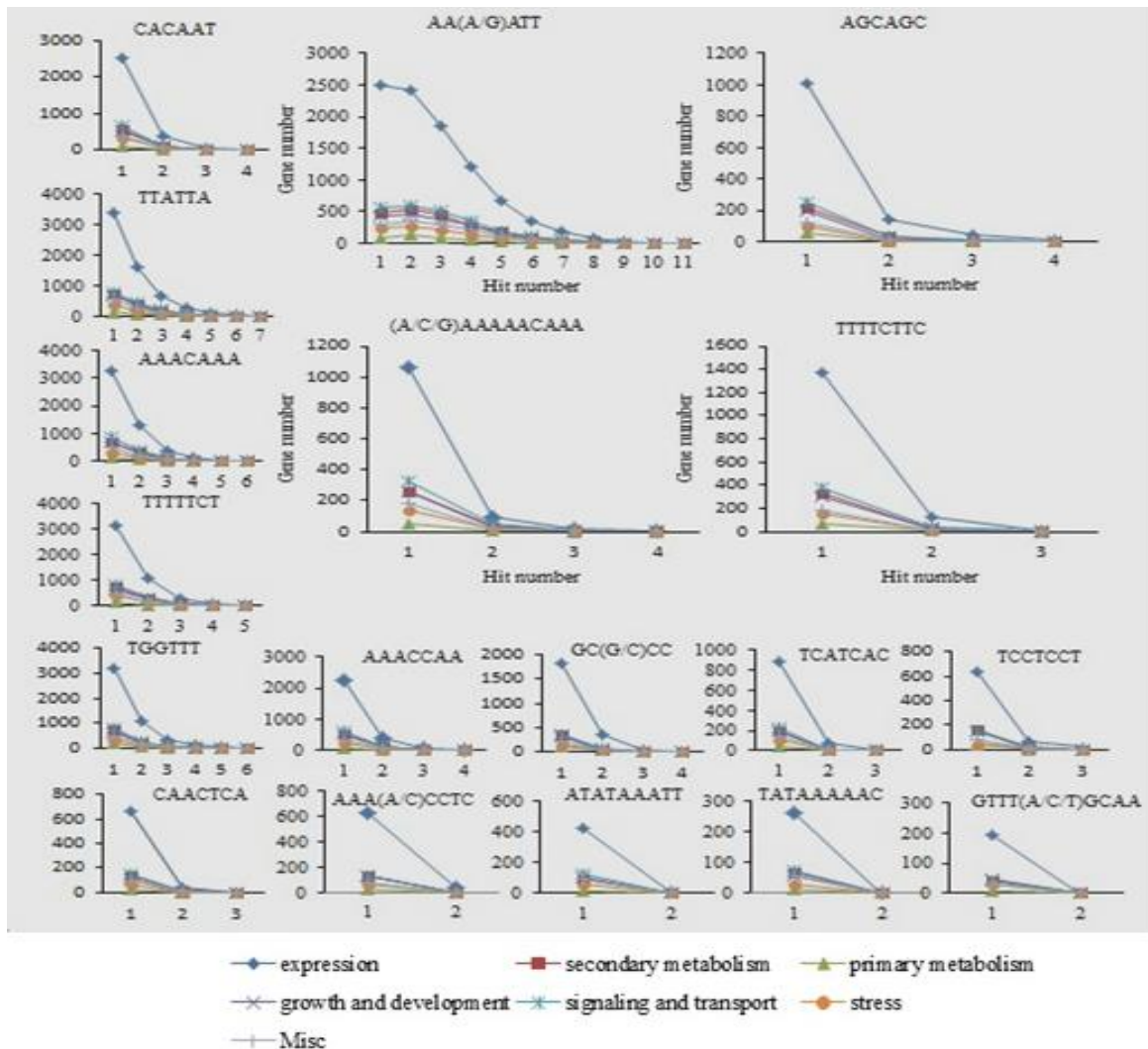


Figure 4. Functional classification of genes that contain AREs. The X axis indicates hit number of elements in promoter and Y axis represents the number of genes in each functional class related to their element hit number. As figure indicates AREs generally tended to occur only in one hit at *Arabidopsis* promoters. In all seven functional classes there was a negative relationship between number of elements and element containing genes. The majority of genes in all AREs were involving in cell expression process; DNA, RNA and protein structural and synthesizing genes. The AREs containing genes with high hit number were removed in all graphs because of low numbers.

Discussion

Divergent motifs of AREs in Arabidopsis genes

Elements activity preserved by selection pressure to eliminate mutation in their sequences, however, degeneracy and variability in number, order and position of cis-regulatory elements prevent this sequence conservation [15]. Presented data revealed that, various sequences (nineteen different motifs) and high frequent occurrences were the most highlight feature of ARE elements in *Arabidopsis* genome. In recent studies, several motifs with a ACGT core, similar to G-boxes, have been identified as promoter landmarks of abscisic acid hormone responsive genes [22-25]. The control process of auxin responsive genes also conducts by a protein family which interacting with three conserved cis-regulatory elements, AuxRR-core, AuxRE and TGA-element by a bias toward

AuxRE element [26-29]. Such a divergence in cis-acting elements, has been reported for gibberellin (GA) responsive genes, the TAACAGA and TCTGTTG motifs which response to GA treatment [18, 29, 30, 31]. Thus motif diversity is a general character of some elements and determines the response of genes containing element to their stimuli. Our data indicated that AREs scattered in *Arabidopsis* genome independent of their length and GC content, therefore, AREs diversity may be involved in specific interactions with transcription factors. In addition, increase in divergent of identical elements can increase tissue specific gene expression [32].

AREs and abiotic stress

Diagnosis of interaction between AtMYB2 transcription factor and TGGTTT element revealed that TGGTTT sequence plays critical role in promoter regions of stress-

responsive genes [11]. Besides this attachment, there are additional evidences to address common function of anaerobic responsive elements in other abiotic stresses. MYB protein family is the largest transcription factor family in *Arabidopsis* that participate in various cell processes [33, 34]. Among all MYB proteins, the AtMYB2 acts as transcriptional activator of abscisic acid (ABA) and ethylene (ET) hormones in stress conditions [33, 35] and also manages the expression of some drought and salt-responsive genes [11, 34, 36]. ABA and ET hormones induce gene expression involved in water logging condition; a situation which the oxygen availability in plant cell decreases rapidly and include induction of fermentation pathway to produce ATP [37-39]. Although, no TFs have been reported to bind AREs except AtMYB2, but some of MYB family and AtMYB2 are immediate target of regulatory proteins [34]. In addition, the similarity of ARE sequences including TTATTA, ATATAAATT, TATAAAAAC and CACAAT to core promoter elements (TATA box and CAAT box) [9] may be a reason that a group of undiscovered TFs could affect or bind to each one of AREs. In general, TATA-box is essential cis-regulatory element for expression of all genes and may create the complexity of the interrelationship among the environmental stresses [40, 41]. It seems that AREs have a propensity to be involved in regulatory networks of abiotic stress and plant responses to anaerobic stress may mediate by ABA and ET hormones. Therefore, regulatory pathways of plant hormones and AREs remain as a big mystery in plant responses.

AREs during evolution of genes

Understanding evolution of elements and noncoding regions area fundamental clue for comprehending the evolution of plants phenotypes [15]. Therefore, the evolution of cis-regulatory elements has greatly affected by environmental factors [42]. As an environmental factor, anaerobic condition exerts a negative impact on plants and this stress can be effective through the mode of operation of the downstream cis-regulatory system. The results of previous experiments demonstrated that three AAACCAA, TGGTTT and GC(G/C)CC ARE elements responded to anaerobic situation in *Arabidopsis* and *Maize*, while the other sixteen AREs identified through an *in silico* survey [6-9]. As well as these elements, our analysis determined that three motifs TTCCTGTT, (A/C/G)AAAAACAAA and AGCAGC had positional bias in genes promoter of *Arabidopsis thaliana*. Surprisingly, number of genes with stress-responsive elements and hormone signaling elements (Fig. 5) were similar to number of genes containing three positional significant elements (PSEs) of AREs (the 0.25, 11.35 and 10.42 percent of total *Arabidopsis* genes, respectively). Among all analyzed elements, the MeJA-R element by 31.5% and TCAGAAGAGG motif of SAR elements by 0.09 % were the most and least frequent elements in *Arabidopsis* genome, respectively (Fig. 5). Positional conservation of elements in specific regions of promoter could create specific biological function for elements [43-45]. Therefore, our judgment suggests that the significant bias

and low percentage of genes containing PSEs may point towards biological importance of them related to other elements. In addition, AREs generally tended to occur only in one hit at *Arabidopsis* promoters (Fig. 4). Kafri et al. (2005) [46] indicated that there is a negative correlation between the number of gene regulatory elements and dispensability of them and it may remark that the functional conservation of AREs have disrupted in genes with one hit during the evolution. This means that although AREs by one hit can be effective on large number of genes, but ubiquitous and random distribution in genome may scale down their effectiveness on both transcriptional level and tissue specific or general gene expression [43, 47]. As exposed in Figure 4, the function of majority of AREs containing genes were observed in genes which involving in expression, signaling and transport, secondary metabolism and growth and development, but only 210 genes of *Arabidopsis* responded to low-Oxygen stress [4]. These data confirm our suggestion that AREs activity may have been erased during evolution. In an overall view, low water availability has a large part of ongoing evolution among abiotic factors involved in shaping of plant evolution or the flora on Earth [48].

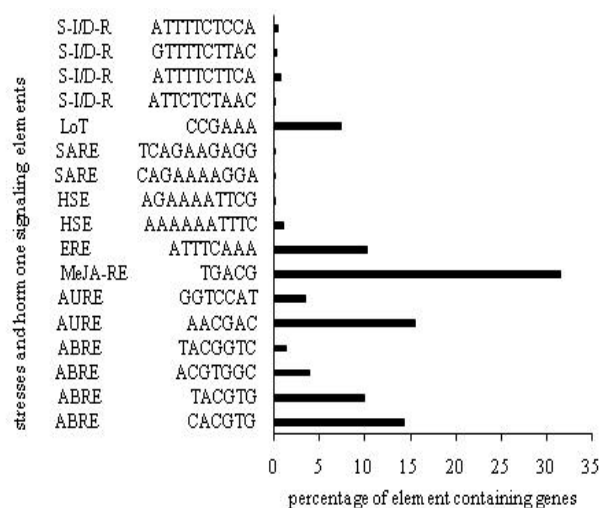


Figure 5. The percentage of stresses and hormone signaling elements containing genes in *Arabidopsis* genome. Percent of all elements were calculated by Promomer tool. Genes with three anaerobic PSEs depicted in Fig. 2 had a low and a similar frequency like stresses and hormone signaling elements. The ABRE, AURE and ERE denote Abscisic acid, Auxin and Ethylene responsive elements respectively, and MeJA-RE, HSE, SARE, LoT, S-I/D-R are abbreviation of Methyl Jasmonate, Heat Shock, Salicylic Acid, low-temperature and Stress inducible/defense-related elements respectively.

It could be assumed that the divergence of AREs in contrast to other cis elements would have originated of two concepts; first, the atmosphere of early earth and second, the present anaerobic stress. By increasing of O₂ during the earth evolution [1] the first plants on soil had shifted from anaerobic to aerobic life. Currently, more than 70% of total cell proteins are anaerobic-related polypeptides in

response to anaerobic stress [3]. It seems reasonable to assume that early anaerobic responsive elements may retire during evolution of plant genes and demand for new anaerobic responsive elements may have risen in new natural environment. Finally, we introduced the TTCCCTGTT, (A/C/G)AAAAACAAA and AGCAGC as cis regulatory elements involved in anaerobic stress among all nineteen AREs, although, the AAACCAA, TGGTTT and GC(G/C)CC elements validated experimentally [10, 11].

Conclusions

Our data demonstrated that anaerobic responsive elements were the most widespread cis-regulatory element in Arabidopsis genome. Although, nineteen candidate elements indicated high divergence and frequency in promoter region of Arabidopsis genes, but microarray analysis revealed that only 210 genes express in anaerobic condition. On the other hand, gradual increase of O₂ in the early atmosphere has revolutionized the development of first land plant. It could be assumed that all nineteen AREs are not indispensable in response to anaerobic conditions and TTCCCTGTT, (A/C/G)AAAAACAAA and AGCAGC positional significant elements play a key role in anaerobic stress. In addition, the affinity of AtMYB2 transcription factor to TGGTTT elements and similarity of TTATTA, ATATAAATT, TATAAAAAC and CACAAT elements to TATA box and CAAT box would be empirical evidences that AREs participate in response to a wide range of abiotic stresses such as anaerobic, drought and salt by mediation of ABA and ET hormones. At the end, future studies of all anaerobic elements should also continue to focus on transcriptional regulatory network in response to anaerobic condition in order to substantiate AREs activity. This will require the identification of TF proteins and molecular mechanisms involved in abiotic stresses.

Acknowledgement

This study was supported by Department of Biotechnology, Faculty of New Technologies Engineering Shahid Beheshti University. The authors also thank Botany Array Resource (BAR database) for easy-to-use web-based data-mining tools.

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