

**DIFFERENTIAL EXPRESSION PROFILES OF
APETALA2/ETHYLENE RESPONSIVE FACTOR (AP2/ERF)
GENE FAMILY IN EGYPTIAN SUGARCANE (*SACCHARUM
SPONTANEUM* L.) IN RESPONSE TO PHYTOHORMONES AND
A BIOTIC STRESS**

Mohamed Ewas

E-mail: mohamed_ewas82@yahoo.com

The APETALA2/Ethylene-Responsive Factor (*AP2/ERF*) gene family is a transcription factors that is particular to plants. *AP2/ERFs* plays essential roles in growth and development regulation, biotic and abiotic stressors tolerance, and responding to plant hormones. However, information on the roles of *AP2/ERF* gene family in Egyptian sugarcane (*Saccharum spontaneum*) is lacking. In the current study, a genome-wide analysis was performed to identify the *AP2/ERF* genes in *S. spontaneum*. Using bioinformatics techniques, 288 *SsAP2/ERF* have been acquired in total. They were classified into five sub-families based on phylogenetic study, with 14 *AP2*, 105 *DREB*, 4 *RAV*, 143 *ERF*, 1 *Soloist*, and 21 *ANT*. Gene structure, chromosome localization and conserved domain were investigated for *SsAP2/ERF* genes. As opposed to its homologue genes, which more often express in nodes and buds, tissue-specific expression analysis demonstrated that *SsAP2/ERF* genes were numerous and expressed ubiquitously in all examined tissues, with slightly higher levels in roots followed by leaves, and flowers. Interestingly, 6 *SsAP2/ERF* genes that contains two *AP2* domains exhibited diverse expression patterns in response to phytohormones including abscisic acid, gibberellic acid, salicylic acid, and methyl jasmonate along with various abiotic stresses such as drought, high salinity, high or low temperature. Concisely, results of this research provide a deep insight towards further functional exploration of the *SsAP2/ERFs* in response to phytohormone and abiotic stress with the eventual goal of developing crop production.

Keywords: expression profiles, *AP2/ERF* family, sugarcane, phytohormones, abiotic stress

INTRODUCTION

Egyptian sugarcane *Saccharum spontaneum* L. is regarded as one of the world's earliest cultivated crops. Egypt is known to be one of the oldest native ranges of this subspecies (Gaber et al., 2009). Its habitat extends on the border of water streams within the Egyptian deserts and Nile delta (Abd El-Gawad and El-Amier, 2017). Commercially grown in tropical and subtropical areas. When favorable environmental conditions for the crop are present, it is the most formative crop (Gaber et al., 2009 and FAO and UN-Water, 2021). Almost 80% of sugar and 40% of ethanol are produced globally using sugarcane (*Saccharum spp.*), a traditional C₄ crop with the highest photosynthetic rates of any crop (Lam et al., 2009; Zhang et al., 2013 and Li et al., 2020). The three major species of the genus *Saccharum* are *S. spontaneum*, *S. officinarum*, and *S. robustum*, according to standard classification (Irvine, 1999). A hybrid between *S. spontaneum* and *S. officinarum* led to the development of the recent sugarcane cultivar. The original species for research on sugarcane are thought to be *S. spontaneum* and *S. officinarum*. However, the only autopolyploid with allele-defined genome data accessible in *Saccharum* is *S. spontaneum* AP85-441, which was developed from the anther of octoploid SES208 (Moore et al., 1989 and Zhang et al., 2018). According to assumptions, *S. officinarum* contributed to the genetic basis of the *Saccharum* hybrid's high sugar content, and *S. spontaneum* to its environmental stresses including biotic and abiotic stresses (Roach, 1972). In Egypt, *S. spontaneum* spread as wild plants in Egyptian deserts, which are characterized by various harsh environmental stresses, including drought, heat, salinity, and other stressors (Zahran et al., 2016). It is extremely harsh to correctly identify all members of some gene families based solely on sequence consideration, particularly for the massive gene families, such as *AP2/ERF*, because of the possible collapsing of homologous sequences that happened during the arrangement of the tetraploid *S. spontaneum* genome (Li et al., 2020). Inclusive understanding a gene family's molecular architecture and evolutionary history in a plant species is the first step in the direction of comprehending the physiological functions and metabolic processes involved in various growth phases (Zhang et al., 2016). Recent research has shown that most plant species included at least five members of the largest gene family known as *AP2/ERF*.

One of the biggest gene families, the *APETALA2*/Ethylene-Responsive Factor (*AP2/ERF*) gene family encodes transcription factors (TFs) unique to plants. The *AP2/ERF* domain, which has between 60 and 70 amino acids and participates in DNA binding, is what distinguishes the *AP2/ERF* superfamily (Guo et al., 2016). The *AP2/ERF* superfamily is further classified into the *AP2* (*APETALA2*), *ERF* (Ethylene Responsive Factors), *Soloist*, *RAV* (Related to

ABI3/VP), and *DREB* (Dehydration Responsive Element Binding) sub families (Sakuma et al., 2002; Wessler, 2005; Nakano et al., 2006 and Liu et al., 2013). Members of the *AP2* subfamily contain several *AP2/ERF* domains or absence conserved *WLG* motifs inside the *AP2/ERF* domains. TFs of the *RAV* subfamily contain individual *AP2/ERF* and *B3* domains, while *DREB* and *ERF* subfamilies have single *AP2/ERF* domain, the rest of TFs are assigned as soloist (Sakuma et al., 2002). The members of the *AP2/ERF* gene family are crucial in controlling how plants develop and how well they can withstand biotic and abiotic stressors (Liu et al., 2013; Schmidt et al., 2013 and Jiang et al., 2014). The *AP2/ERF* gene family has been discovered in many diverse plants, including *Arabidopsis* and rice (Nakano et al., 2006), maize (Liu et al., 2013), sorghum (Yan et al., 2013), soybean (Zhang et al., 2008), and foxtail millet (Lata et al., 2014). However, no study has been carried out to identify or characterize the members of gene family *AP2/ERF* in *S. spontaneum*. In the current study, bioinformatic techniques were used to identify *SsAP2/ERF* genes in Egyptian sugarcane *S. spontaneum* and created a phylogenetic tree. Using drawing tools, most of the *SsAP2/ERF* genes were localized to chromosomes, and duplication processes were also examined. Via published RNA sequencing, the expression patterns of *AP2/ERF* genes were revealed. Finally, quantitative real-time polymerase chain reaction (qRT-PCR) was applied to measure the expression level of the only 6 *AP2/ERF* genes contain two *AP2* domains in response to a biotic stress including drought, high-salt stress, heat shock and cold along with hormone stresses including exogenous abscisic acid (ABA), gibberellic acid (GA_3), salicylic acid (SA), and methyl jasmonate (MeJA). These findings will be beneficial for prospective research on the *AP2/ERF* family in various plant species.

MATERIALS AND METHODS

1. Plant Material and Sequence Database Searches

Samples of Egyptian sugarcane *S. spontaneum* were taken from the Nile delta habitat where it grows along the edge of streams (Location data 31°39'16.9"E). Following Boulos (2005), the samples were identified. To gather every *S. spontaneum* *SsAP2/ERF* gene member, numerous database searches were conducted. The sugarcane genome hub. Tripal database infrastructure (<https://sugarcane-genome.cirad.fr/>), TAIR (The *Arabidopsis* Information Resource) (<http://www.arabidopsis.org/>), and the plant TF database (<http://planttfdb.cbi.pku.edu.cn/>) were used to acquire the *AP2/ERF* sequences of *S. officinarum*, *Arabidopsis thaliana*, *S. spontaneum*, (Jin et al., 2017). The genomic information for *S. bicolor* as well as additional species were gathered from NCBI (<https://www.ncbi.nlm.nih.gov/>) and Phytozome

(<https://phytozome.jgi.doe.gov/pz/portal.html>). Zhang lab produced the *S. spontaneum* genome data; its GenBank accession number is QVOL00000000. (Zhang et al., 2018).

2. Identification of AP2/ERF in *S. spontaneum*

Using the BLASTP program and default parameters, an HMM profile of the AP2/ERF (PF03106) was derived from the Pfam protein family database (<http://pfam.xfam.org/>) (Finn et al., 2016). This profile was used to recognize probable AP2/ERF from the *S. bicolor* genome sequence (Ling et al., 2011). The chosen sorghum AP2/ERFs were then utilized as query sequences in BLASTP searches against the *S. spontaneum* predicted sequences. The HMM profile of AP2/ERFs domains was used as the query to search all possible proteins, and the sequences with *E*-values lower than $1e^{-10}$ were chosen for additional study. Subsequently using the online ExPASy-ProtParam programme (<http://web.expasy.org/protparam/>), both the chemical and physical features of the putative *SsAP2/ERFs* were determined. The genes that were wrongly predicted were manually annotated.

3. Multiple Sequence Alignment, Phylogenetic Analysis and Classification of Sugarcane AP2/ERFs

DNAMAN was used to perform numerous sequence alignments on 288 putative *SsAP2/ERF* proteins. Using MUSCLE in MEGA 7.0's default parameters, the domain sequences of *SsAP2/ERFs* and *AtAP2/ERFs* were aligned (Kumar et al., 2016). Using MEGA 7.0, the NJ technique, the Poisson model, pairwise deletion, and the bootstrap test replicated 1,000 times, the phylogenetic tree based on the alignments was created. The previously reported categorization of *SsAP2/ERFs* and the multiple sequence alignment were used to categorize *SsAP2/ERFs* into several groups and subgroups. (Ross et al., 2007; Rushton et al. 2010 and Xu et al., 2016).

4. Exon-Intron Structure of *SsAP2/ERF* Genes

Diagrams were collected via the online tool Gene Structure Display Server (<http://gsds.cbi.pku.edu.cn/>), and the exon-intron structures of the sugarcane AP2/ERF genes were established depending on their coding sequence alignments and corresponding genomic sequences (Hu et al., 2015).

5. Chromosomal Locations and Collinearity Analysis for All *SsAP2/ERFs*

The database of the *S. spontaneum* genome was used to determine the precise position of *SsAP2/ERFs* on the chromosomes. The Multiple Collinearity Scan toolbox (MCScanX) and the BLASTP program (*E*-value $< 1e^{-5}$) were utilized to examine the duplication pattern for each *SsAP2/ERF* gene (Wang et al., 2012).

6. Plant Growth and Stress Treatments

For gene expression profiling investigation, rhizomes of Egyptian sugarcane plants (*S. spontaneum*) were planted at Tanta University's experimental greenhouse in Gharbia, Egypt, during the 2021/2022 season. Ammonium nitrate was added in the amount of 40 kilogram per feddan. Irrigation practices were administered at 10–15-day intervals throughout the growing season, except for August and September, which were at 3–5-day intervals. Two-month-old uniformly grown *S. spontaneum* plants were exposed to various stressors or hormone treatments. Salt stress was generated by watering the plants with a 200 mM NaCl solution. Drought stress was reproduced by placing detached leaves on filter paper in 70% relative humidity at 25°C. Cold or heat stress conditions were induced by transplanting the plants to a growth chamber and keeping them at 4°C or 40°C, respectively. The wounding was done by pinching the leaves with forceps. To address hormones and oxidative stress, 100 µM ABA, 100 µM GA₃, 100 µM SA, and 100 µM MeJA were sprayed directly onto *S. spontaneum* plants. Following each treatment, leaves from three distinct plants (three biological replicates) were collected and promptly frozen in liquid nitrogen before being held at 80°C until RNA was extracted. Salt stress was generated by watering the plants with a 200 mM NaCl solution. Drought stress was reproduced by placing detached leaves on large filter paper in 70% relative humidity at 25°C. Cold or heat stress conditions were induced by transplanting the plants to a growth chamber and keeping them at 4°C or 40°C, respectively. To address hormones and oxidative stress, 100 µM ABA, 100 µM GA₃, 100 µM SA, and 100 µM MeJA were sprayed directly onto *S. spontaneum* plants. Following each treatment, leaves from three distinct plants (three biological replicates) were collected and promptly frozen in liquid nitrogen before being held at 80°C until RNA was extracted.

7. Expression Analyses of *SsAP2/ERFs*

The first-strand complementary DNA (cDNA) was generated using 3 µg of total RNA and 200 U of M-MLV reverse transcriptase (from Invitrogen), following the manufacturer's instructions. Total RNA was isolated using Trizol solution (Invitrogen). Using first-strand cDNA as a template, RT-PCR was performed to amplify a 400 bp fragment of each *SsAP2/ERF* gene with 31 cycles. As an additional internal control, the action was amplified for 24 cycles. Using the SYBR Premix Kit F-415 and an AB StepOnePlus PCR equipment from Applied Biosystems, real-time PCR was carried out on an optical 96-well plate. (Thermo Scientific). A relative quantification method⁵⁰ was used to determine relative gene expression. The list of all primers used in this investigation can be found in Table (1).

Table (1). Forward and reverse primers used in the qRT-PCR genes expression studies.

Gene name	Sequence (5' - 3')
<i>SsAP2/ERF-1 - F</i>	ATTTCCGCAACCCATTTCCC
<i>SsAP2/ERF-1 - R</i>	CTGCCAAACCACCACCTTAC
<i>SsAP2/ERF-2 - F</i>	GCGGAGGGGAAGAGTATTGA
<i>SsAP2/ERF-2 - R</i>	ACGGGTACTIONTTCCAGCAGA
<i>SsAP2/ERF-3 - F</i>	TCCACCAGATCCAAACCCAA
<i>SsAP2/ERF-3 - R</i>	GCTTCTTCGGCTGTGTCAA
<i>SsAP2/ERF-4 - F</i>	GATCCCAAAGCCCACCAATC
<i>SsAP2/ERF-4 - R</i>	AAATTAAGCCGAGCCGATGG
<i>SsAP2/ERF-5 - F</i>	TTTCAAATGCGTGGCTCCAA
<i>SsAP2/ERF-5 - R</i>	GACTGCCGTTATGTTGGGG
<i>SsAP2/ERF-6 - F</i>	GGATAAGAAGTCGCAGTGGC
<i>SsAP2/ERF-6 - R</i>	AAGTTCAACACGGCATCACC
<i>Actin - F</i>	TGGAATGGAAGCTGCTGGTA
<i>Actin - R</i>	TTGATCTTCATGCTGCTCGG

RESULTS

1. Identification and Characterization of *AP2/ERF* Gene Family in *S. spontaneum*

In general, 288 genes were identified as agreeable *AP2/ERF* genes in *S. spontaneum*. The expected *SsAP2/ERF* genes (public name and locus ID has been presented in Supplementary Table S1 in specifics) were subsequently chosen depending on the position of chromosome and their family categorization (Table S1). According to the classification, they were divided into 14 *AP2*, 105 *DREB*, 4 *RAV*, 143 *ERF*, 1 *Soloist* and 21 *ANT*. Thirty five *AP2* TFs with a single or two *AP2* domains that were identical with *AP2* domains in double domain groups were categorized into the APETALA2/Ethylene Responsive Factor gene family (*AP2/ERF*), while 4 genes include B3 type domain were categorized as *RAV* gene family. Only one domain from the *ERF* subfamily, which was further subdivided into the *ERF* and *DREB* subgroups, was present in 248 genes. Also, a particular gene known as *SsAP2/ERF-288* resembled other relatives that belonged to the *Soloist* subgroup (Table S1). In the 20 chromosomes of the *S. spontaneum*, the distribution of *SsAP2/ERF* genes was shown to be uneven (Fig. 1). The chromosomes with the greatest and smallest number of *AP2/ERFs* were 2 and 16 (30 genes) and 5 (9 genes), respectively.

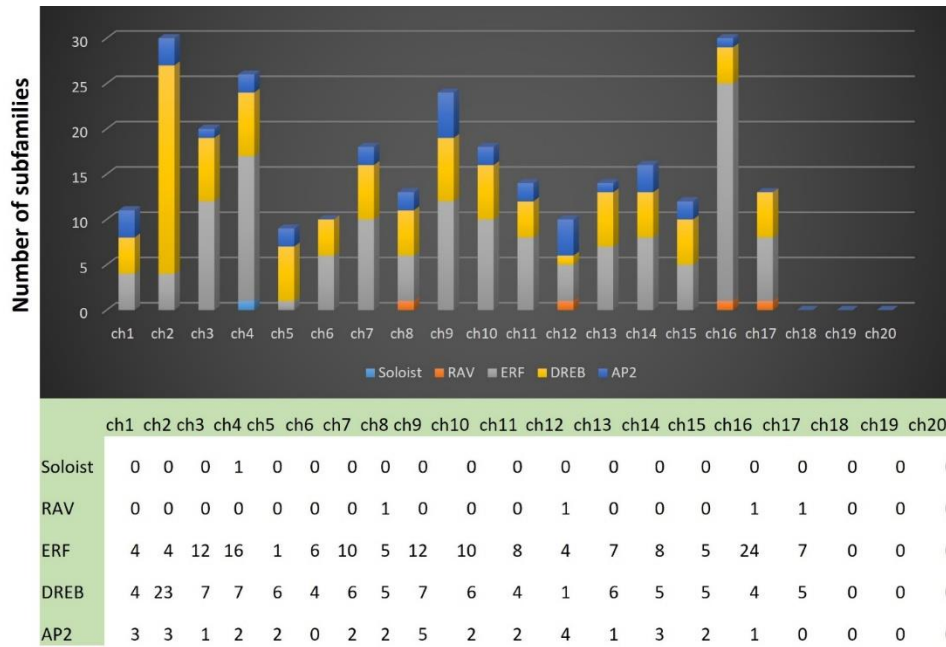


Fig. (1). Chromosome-wise distribution of 288 *AP2/ERF* genes on 20 chromosomes of *Saccharum spontaneum*.

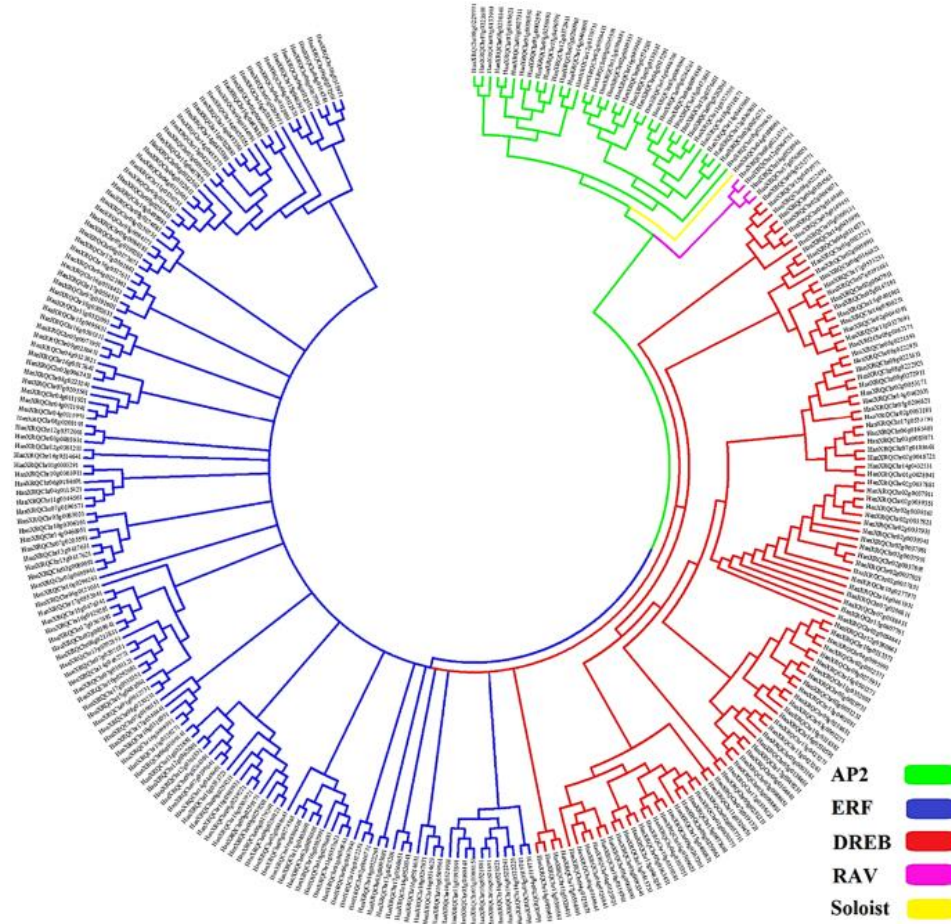
2. Phylogenetic Relationships and Gene Structure Analysis of *SsAP2/ERF*

Based on the numerous correlations of all *SsAP2/ERF* with *Arabidopsis AP2/ERF* genes, phylogenetic analysis was carried out to evaluate the evolutionary links of the *SsAP2/ERF* genes. *SsAP2/ERF* family proteins were used to create an unrooted phylogenetic tree (Fig. 2). *ERF* clades were also split up into 10 groups. The *ERF* families could be further separated into a subgroup of *DREB* and the *ERF* families, much like the *Arabidopsis* assortment criteria (Guo et al., 2016).

The following six groups (V-X) were the *ERF* subgroups, and the remaining four groups (I-IV) belonged to *DREB* (Fig. 3). Among 35 *AP2* TFs only 6 *AP2/ERF* genes contain two *AP2* domains (Fig. 4). The analysis of phylogenetic tree rearranged the six *AP2/ERF* genes according to the degree of their structure similarity or divergence with each other. The examination of *SsAP2/ERF* proteins for conserved motifs revealed that each of the proteins has two *AP2* structural domains (Motif 1 and Motif 2, or *AP1* and *AP2*) that comprise *AP2/ERF* domain sequences. The *SsAP2/ERF* gene motifs on the same branch are comparable in number, type, and arrangement, and the functional variations in

tomato *SsAP2/ERF* genes may result from variations in the distribution of conserved motifs (Fig. 4).

Fig. (2). An unrooted phylogenetic tree of AP2/ERF family proteins in *Saccharum spontaneum*. The complete sequences of 288 AP2/ERF family proteins identified



in this study were aligned by ClustalX2.1 and the phylogenetic tree was constructed using the neighbor-joining method with MEGA7.0 software.

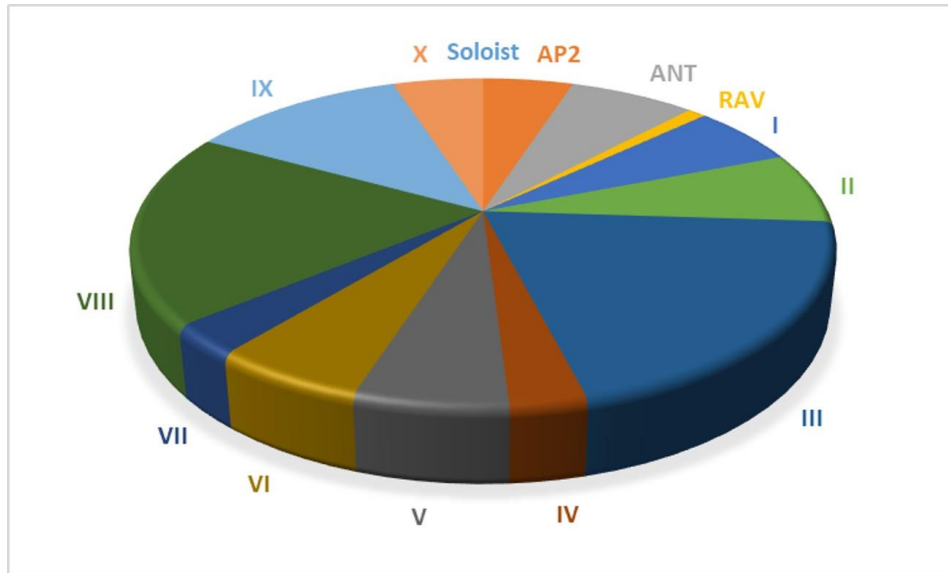


Fig. (3). The percentage of genes belonging to different groups *SsAP2/ERF*.

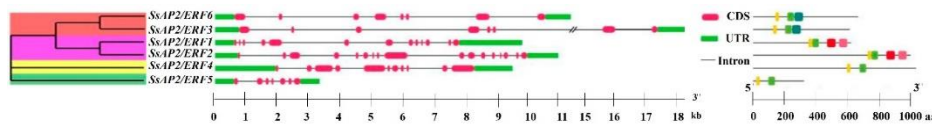


Fig. (4). Gene structure of the *AP2/ERF* gene family in *Saccharum spontaneum*. The asterisks indicate motifs shared by all *SsAP2/ERFs*.

3. Synteny Analysis of *SsAP2/ERF* Genes

To evaluate the molecular evolutionary links between species, synteny analysis is a crucial analytical method in comparative genomics (Zhao and Schranz, 2017). The *AP2/ERF* gene was more homologous on Egyptian sugarcane *S. spontaneum* and *S. officinarum*, likely due to their close kindred, according to a homology analysis of the *SsAP2/ERFs* between *S. spontaneum* and other species, as shown in Fig. (5). Interestingly, *SsAP2/ERF2* and *SsAP2/ERF5* correspond to two TF pairs found in the respective organisms *Arabidopsis thaliana* and *S. officinarum*. Even in the presence of gene duplications or

chromosomal rearrangements, *SsAP2/ERF* synteny analysis revealed substantial collinearity.

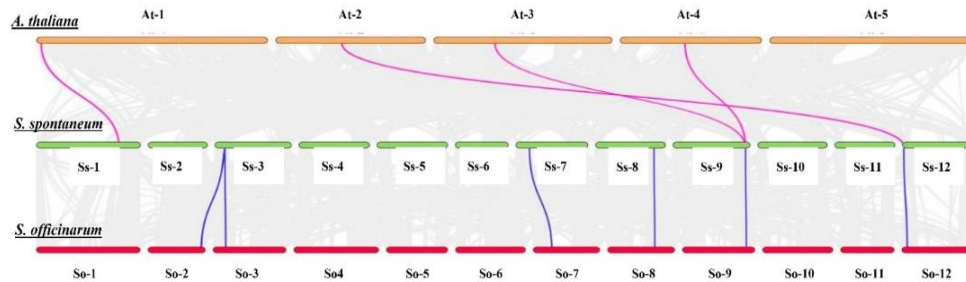


Fig. (5). Syntenic relationships between homologous *SsAP2/ERFs* of *Saccharum spontaneum* and other species including *Arabidopsis thaliana* and *Saccharum officinarum*.

4. Tissue-Specific Expression Analysis of *SsAP2/ERF* Genes in *S. spontaneum*

RNA was extracted from roots, nodes, internodes, buds, leaves and flowers. Using qRT-PCR, the expression of all *SsAP2/ERF* genes in the Egyptian sugarcane *S. spontaneum* was identified. The *SsAP2/ERF* gene expression profile showed that there were differences in the transcription level of the six *SsAP2/ERF* genes in different tissues of *S. spontaneum*. The expression of *SsAP2/ERF4* was higher than other *SsAP2/ERF* genes, as shown in Fig. (6). As opposed to its homologue genes, which more often express in nodes and buds, tissue-specific expression analysis demonstrated that *SsAP2/ERF* genes were numerous and expressed ubiquitously in all examined tissues, with slightly higher levels in roots followed by leaves, and flowers (Fig. 6), suggesting their fullest potential function outside of nodes and buds. The tissue-specific expression analysis of *SsAP2/ERF* genes in *S. spontaneum* revealed that each of *SsAP2/ERF1*, *SsAP2/ERF2* and *SsAP2/ERF4* were expressed in roots followed by leaves, and flowers higher than the remain tissues including buds, nodes and internodes. On the other hand, the transcripts of *SsAP2/ERF3*, *SsAP2/ERF5* and *SsAP2/ERF6* varied from one tissue to another with different levels (Fig. 6).

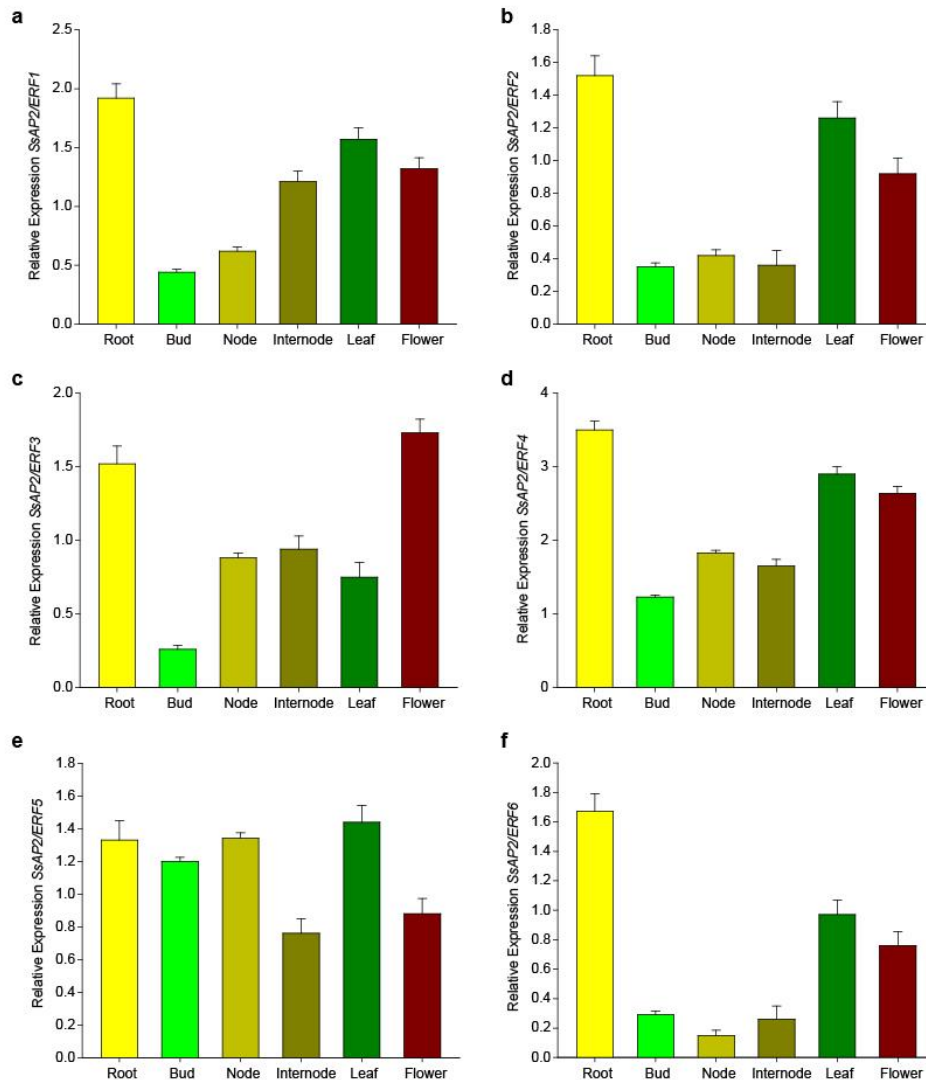


Fig. (6). Tissue-specific expression analysis of *SsAP2/ERF* genes in *Saccharum spontaneum*. **a.** Expression patterns of *SsAP2/ERF1*; **b.** Expression patterns of *SsAP2/ERF2*; **c.** Expression patterns of *SsAP2/ERF3*; **d.** Expression patterns of *SsAP2/ERF4*; **e.** Expression patterns of *SsAP2/ERF5*; **f.** Expression patterns of *SsAP2/ERF6* using qRT-PCR (relative to *actin*).

5. Differential Expression Profiles of *SsAP2/ERF* Genes in Response to Abiotic Stress

To better understand and validate the expression of these identified *AP2/ERF* genes, the six *SsAP2/ERF* genes that contains two AP2 domains were picked to assist scientists in detecting their transcription levels in the leaves of 2-months-old *S. spontaneum* in response drought, high salt, cold and heat stresses using qPCR (Fig. 7). The *SsAP2/ERF* genes were significantly induced by some of the stress conditions. Under drought stress, the transcripts of all *SsAP2/ERF* genes were induced varyingly (Fig. 7a). Under drought stress conditions, the gene *SsAP2/ERF4* had the highest expression, followed by *SsAP2/ERF1*, then *SsAP2/ERF2*, and the gene *SsAP2/ERF5* had the lowest expression compared with their expression levels under normal conditions (Fig. 7a). Under high salt stress (200 mM NaCl), the expression of *SsAP2/ERF1*, *SsAP2/ERF2*, *SsAP2/ERF3*, *SsAP2/ERF4*, *SsAP2/ERF5* and *SsAP2/ERF6* increased approximately up to 2, 2.5, 3, 4, 2.5 and 6-fold, respectively (Fig. 7b). Interestingly, subjecting *S. spontaneum* plants to cold stress (4°C) resulted in the accumulation of *SsAP2/ERF* transcripts as follow, *SsAP2/ERF1*, *SsAP2/ERF3*, *SsAP2/ERF4* and *SsAP2/ERF6* up to 20, 18, 16, 16-fold, while transcripts of both *SsAP2/ERF2* and *SsAP2/ERF5* genes did not show any significant response to cold stress (Fig. 7c). On the other hand, heat stress (40°C) gradually up-regulated the expression of *SsAP2/ERF1*, *SsAP2/ERF2*, *SsAP2/ERF3*, *SsAP2/ERF4*, *SsAP2/ERF5* and *SsAP2/ERF6* by approximately 4, 3, 5, 4, 2 and 1.5-fold, respectively (Fig. 7d). In general, among the *SsAP2/ERF* gene family, the genes most responsive to abiotic stress are *SsAP2/ERF1*, *SsAP2/ERF2*, and *SsAP2/ERF4*.

6. Differential Expression Profiles of *SsAP2/ERF* Genes in Response to Phytohormones

To investigate the hormonal response of *SsAP2/ERFs*, four major hormones were chosen for this study including ABA, GA₃, SA, and MeJA. The current study's findings revealed, in general, a rapid response of *SsAP2/ERFs* genes to the applied hormones. As compared to other applied hormones, the highest transcription levels of *SsAP2/ERF* genes were associated with the ABA treatment. Furthermore, the transcripts of *SsAP2/ERF1*, *SsAP2/ERF2*, *SsAP2/ERF3*, *SsAP2/ERF4*, *SsAP2/ERF5* and *SsAP2/ERF6* rapidly increased up to 12, 8, 6, 10, 5 and 6-fold, respectively within 12 hours in response to ABA (100 µM) (Fig. 8a). A similar pattern was seen for transcript induction by SA (100 M), which resulted in 2, 1.5, 2, 2.5, 2.5, and 1.5-fold increases in *SsAP2/ERF1*, *SsAP2/ERF2*, *SsAP2/ERF3*, *SsAP2/ERF4*, *SsAP2/ERF5*, and *SsAP2/ERF6* mRNA accumulation (Fig. 8b). Regarding GA₃ treatment, the response of

SsAP2/ERF genes varied significantly from one another, with *SsAP2/ERF3*, *SsAP2/ERF5*, and *SsAP2/ERF6* being markedly elevated while *SsAP2/ERF1*, *SsAP2/ERF2*, and *SsAP2/ERF4* being notably reduced (Fig. 8c). MeJA (100 M) caused up-regulation of *SsAP2/ERF1*, *SsAP2/ERF4*, and *SsAP2/ERF6* by approximately 3, 2, and 4-fold, respectively, and down-regulation of *SsAP2/ERF2* and *SsAP2/ERF5*, with no significant difference in *SsAP2/ERF3* transcript levels (Fig. 8d). Overall, ABA and SA hormones were more effective than the other hormones (Fig. 8).

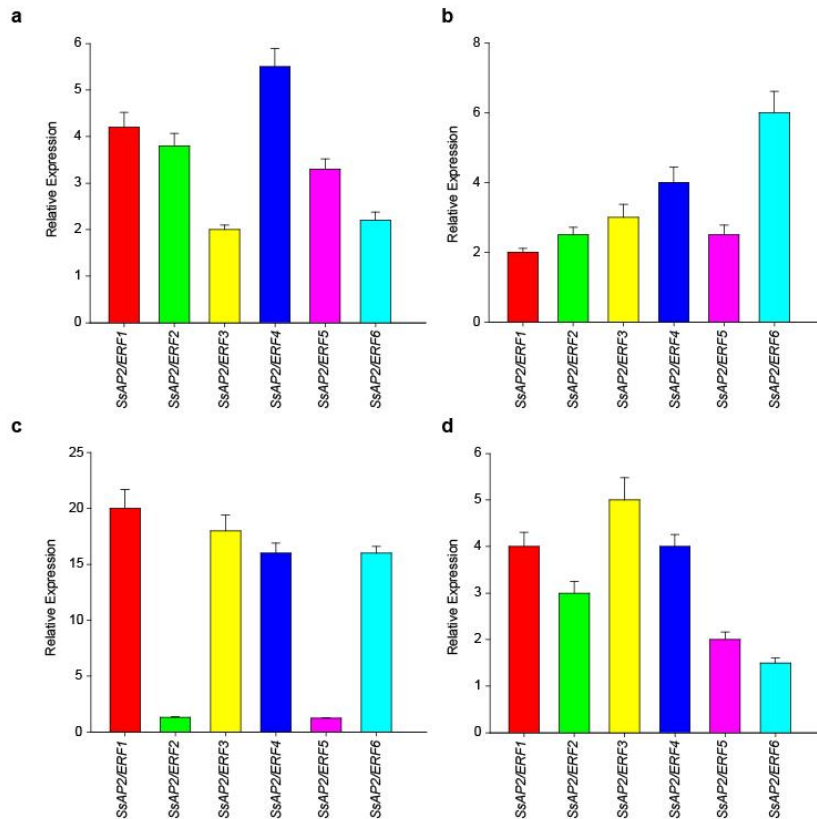


Fig. (7). Expression profiling of *SsAP2/ERF* gene family in response to abiotic stress compared to control using qRT-PCR (relative to *actin*). **a.** Expression profiling of *SsAP2/ERF* gene family in response to drought stress; **b.** Expression profiling of *SsAP2/ERF* gene family in response to salt stress; **c.** Expression profiling of *SsAP2/ERF* gene family in response to heat stress; **d.** Expression profiling of *SsAP2/ERF* gene family in response to cold stress.

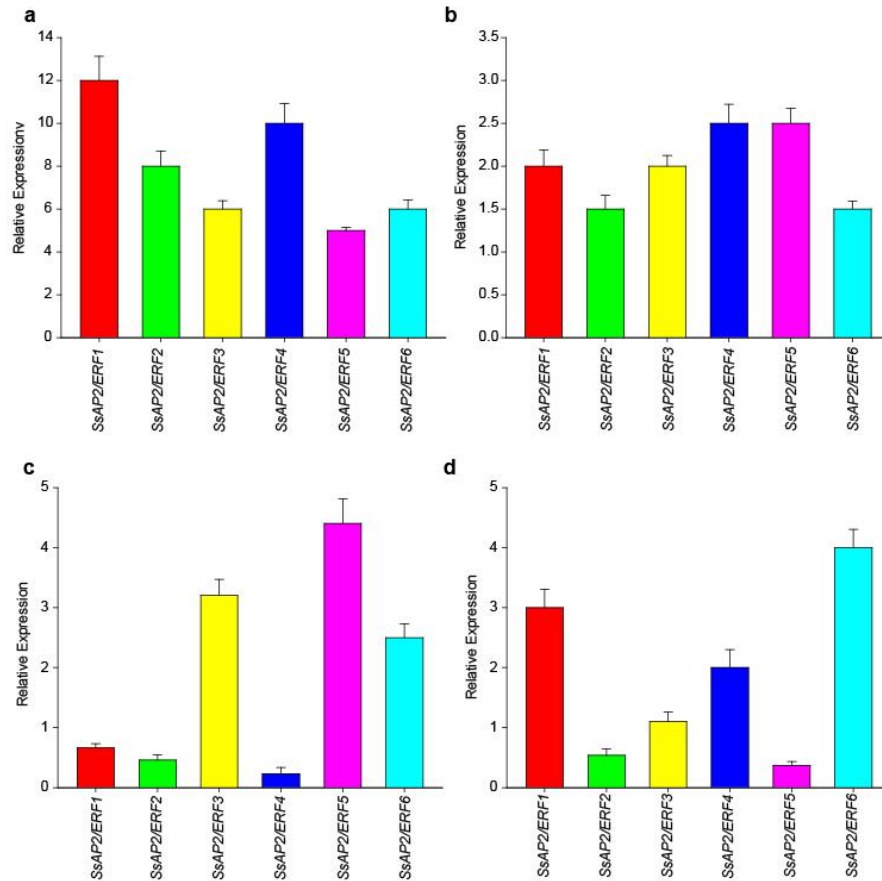


Fig. (8). Expression profiling of *SsAP2/ERF* gene family in response to phytohormones compare to control using qRT-PCR (relative to *actin*). **a.** Expression profiling of *SsAP2/ERF* gene family in response to abscisic acid (ABA); **b.** Expression profiling of *SsAP2/ERF* gene family in response to gibberellic acid (GA₃); **c.** Expression profiling of *SsAP2/ERF* gene family in response to salicylic acid (SA); **d.** Expression profiling of *SsAP2/ERF* gene family in response to and Methyl jasmonate (MeJA).

DISCUSSION

1. Identification of the Egyptian Sugarcane *S. spontaneum* AP2/ERF superfamily

The AP2/ERF superfamily is one of the most extensive collections of TF families in crops. It additionally plays a vital role in the transcriptional control of

environmental stresses including tolerance to heat shock, cold, wounding, drought and salt stressors along with pathogen resistance and intricate developmental processes such as fruit ripening, seed germination, flowering, and leaf senescence (Klucher et al., 1996; Dubouzet et al., 2003; Yang et al., 2011; Schmidt et al., 2013 and Zhu et al., 2014). The *AP2/ERF* gene family in plants has been extensively studied depend on plant genome sequencing (Nakano et al., 2006; Zhang et al., 2008; Sharma et al., 2010; Liu et al., 2013; Yan et al., 2013; Lata et al., 2014; Sun et al., 2014 and Thamilarasan et al., 2014). However, knowledge of the sugarcane *SsAP2/ERF* genes is currently limited. 288 *SsAP2/ERF* genes were discovered from the 87289 (up-regulated and down-regulated genes) identified genes and genomic DNA database (Li et al., 2020) to further study the *AP2/ERF* family in Egyptian sugarcane *S. spontaneum*. Each of them possesses distinguishing characteristics, including at least one conserved AP2/ERF domain. surprisingly, *S. spontaneum* had more *SsAP2/ERF* genes than *Arabidopsis thaliana* (147 genes), *Oryza sativa* (164 genes) and *Zea mays* (210 genes) (Nakano et al., 2006 and Liu et al., 2013). Furthermore, the numbers of certain subfamilies were comparable. The number of AP2 subfamily members in *S. spontaneum* and *Arabidopsis thaliana*, for example, was 14 and 18, respectively, which was half of the number in rice and maize. RAV subfamily counts in *S. spontaneum*, *Hordeum vulgare*, *Arabidopsis thaliana*, *Oryza sativa* and *Setaria italica* were 4, 6, 6, 7, and 5, respectively. The numbers of other subfamilies, on the other hand, were dramatically different. In the *DREB/ERF* subfamily of *S. spontaneum*, for example, 105 genes have been discovered. *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays*, and *Setaria italica* have 122, 131, 163, and 138 genes, respectively. The present study showed that *S. spontaneum* has only 6 *AP2/ERF* genes that contains 2 AP2 domains. The fewer number of *AP2/ERF* family genes in *S. spontaneum* proposed that there may be more additional *SsAP2/ERF* genes present in the undiscovered genomic locales or that the evolutionarily constrained chromosomal duplication occurred in barley (Guo et al., 2016). The function of genes is significantly influenced by the conserved motifs found in TFs (Sakuma et al., 2002). In *Arabidopsis*, fifty conserved domains were found that were not part of the AP2/ERF motif (Nakano et al., 2006). In the current study, Five AP2/ERF protein domains were examined; domain 1 (a portion of the AP2/ERF domain) was found in all gene members, whereas the other domains were found outside the AP2/ERF motif. The importance of these conserved amino acid residues for the *AP2/ERF* subfamily genes involved in various types of physical contact with DNA is likely indicated by their presence (Sakuma et al., 2002).

2. Expression Analysis Suggested SsAP2/ERF Genes May Play Essential Roles During Plant Growth and Development Along With Abiotic stress and Hormone Response

Tissue-specific expressions that results at a specific growth stage can be used to discover genes that play a role in determining the exact characteristics of individual tissues. The expression pattern of 288 *SsAP2/ERF* genes was discovered by RNA sequencing in this work, which helped to analyze the function of the *SsAP2/ERF* genes in *S. spontaneum*. Surprisingly, the *SsAP2-4* gene was also known as Cleistogamy 1 (*Cly1*)/*SsAPETALA2* (*SsAP2*), and it was an ortholog of the *Arabidopsis thaliana* *AP2* (AT4G36920.1), *TOE3* (AT5G67180.1), and the *Oryza sativa* AP2-like gene Os04g0649100 (Nair et al., 2010 and Houston et al., 2013). *Cly1* expression was discovered in the root, leaves and lodicule up to the stamen primordium stage via in situ RNA hybridization (Nair et al., 2010). Another gene, *HvDREB2.2*, commonly known as *Nud* (*Nudum*), regulates covered/naked caryopsis in *Hordeum vulgare* and exhibits expression in the caryopsis 14-day-post anthesis instead of the hulls or leaves (Taketa et al., 2008). *SsAP2/ERF4* showed relatively high transcription levels in roots, leaves, and flowers at two-month-old plants in the current study. Consequently, more complex specificity expression analysis is useful to decipher the role of *SsAP2/ERF* genes.

In their native surroundings, plants experienced negative environmental pressures. They have developed a wide range of molecular strategies to deal with them. In plants, 2 ABA dependent and 2 ABA independent signal transduction pathways are implicated in the genes' responses to drought, high-salt, and high or low temperature stress (Shinozaki and Yamaguchi-Shinozaki, 1997 and Nakashima et al., 2000). The expression profiling of *SsAP2/ERF* genes under various hormones stress unveiled the vital roles of phytohormones including ABA, SA, MeJA and GA₃ in impacting environmental stress. The fact that Egyptian sugarcane *S. spontaneum* is one of the plant species that grows wild in desert environments and is characterized by a variety of adverse environmental conditions such as drought, salinity, and high or low temperature, it requires a distinctive genetic stock that enables it to resist these a biotic stress, including *SsAP2/ERF* gene family. Considering the ongoing climatic challenges, using crop wild relatives to increase genetic diversity and enhance crop adaptation appears to be a promising and sustainable strategy for agricultural development (Kapazoglou et al., 2023). Crop wild close relatives are earlier generations or progenitors of cultivated crop species, as well as other nearby relatives via evolutionary history which can naturally cross with cultivated species, occasionally using supporting methods (Choudhary et al., 2017). To investigate the molecular mechanisms underlying plant environment adaptation under abiotic

stress and hormone response, gene expression analysis of *SsAP2/ERFs* should be helpful. The function of the differentially expressed genes between *S. spontaneum* and *S. officinarum* needs to be further examined in relation to abiotic stress and hormone response.

CONCLUSION

The present study sought to discover and describe the *S. spontaneum* AP2/ERF TFs and 288 *SsAP2/ERF* genes were identified after a thorough genomic search. All their information was verified by looking at entire cDNA or EST sequences. Analysis and comparisons were made of the chromosomal locations, exon-intron structures, conserved motif combinations, and phonological relationships of *SsAP2/ERFs*. Based on the amount of AP2 domains and probabilistic functions, *SsAP2/ERFs* may be divided into five subgroups. In addition to being evaluated in relation to heat, cold, salt, and drought stress, the gene expression of the six *SsAP2/ERF* genes contains two AP2 domains in various tissues (including roots, nodes, internodes, buds, leaves and flowers) was also examined. The role of many *SsAP2/ERF* genes in plant development and stress response was discovered, and they might be investigated further. This study unveils for the first time the regulation, structure, evolution, and transcription levels of the *SsAP2/ERF* family, which simplifies the process of the *SsAP2/ERF* gene function analysis and develops a basis for a deeper comprehension of the molecular mechanisms underlying plant development and physiological stress processes in *S. spontaneum*.

ACKNOWLEDGEMENTS

The author would like to thank Prof. J. Zhang for his technical support to the research; Dr. Eman khames and Dr. Mohamed Nady for their suggestions.

REFERENCES

- Abd El-Gawad, A. and Y.A. El-Amier (2017). Anatomical features of three perennial swampy plants of Poaceae, grown on the water stream banks in Nile Delta, Egypt. *J. Med. Bot.*, 1: 58-64.
- Boulos, L. (2005). In: 'Flora of Egypt'. Vol. 1, 2, 3, and 4. All Hadara Publishing, Cairo, Egypt.
- Choudhary, M., V. Singh, V. Muthusamy and S. Wani (2017). Harnessing crop wild relatives for crop improvement. *LS Int. J. Life Sci.*, 6: 73.
- Dubouzet, J.G., Y. Sakuma, Y. Ito, M. Kasuga, E.G. Dubouzet, S. Miura et al. (2003). *OsDREB* genes in rice, *Oryza sativa* L., encode transcription

- activators that function in drought-, high-salt- and cold-responsive gene expression. *Plant Journal for Cell and Molecular Biology*, 33 (4): 751–763.
- FAO and UN-Water (2021). Progress on level of water stress. Global baseline for SDG 6 Indicator 6.4.2: Level of water stress: freshwater withdrawal as a proportion of available freshwater resources. Rome, FAO and UN-Water. Available online: <http://www.fao.org/3/cb6241en/cb6241en.pdf>
- Finn, R.D., P. Coghill, R.Y. Eberhardt, S.R. Eddy, J. Mistry, A.L. Mitchell et al. (2016). The Pfam protein families database: towards a more sustainable future. *Nucleic Acids Res.*, 44: 279–285.
- Gaber, A.A. and M.F. Abo El-Fatth (2009). Evaluation of some imported wild cane genotypes imported from USA under Egyptian conditions. *Alex. Scie. Exch. J.*, 30: 128-135.
- Guo, B., Y. Wei, R. Xu, S. Lin, H. Luan, C. Lv, X. Zhang, X. Song and R. Xu (2016). Genome-wide analysis of APETALA2/Ethylene-Responsive factor (*AP2/ERF*) gene family in barley (*Hordeum vulgare* L.). *PLoS One*, 11 (9): e0161322.
- Houston, K., S.M. McKim and J. Comadran (2013). Variation in the interaction between alleles of HvAPETALA2 and microRNA172 determines the density of grains on the barley inflorescence. *Proc. Natl. Acad. Sci. USA*, 110: 16675–16680.
- Hu, B., J. Jin, A.Y. Guo, H. Zhang, J. Luo and G. Gao (2015). GSDS 2.0: an upgraded gene feature visualization server. *Bioinformatics*, 31: 1296–1297.
- Irvine, J.E. (1999). *Saccharum* species as horticultural classes. *Theor. Appl. Genet.*, 98: 186–194.
- Jiang, Q.Y., Z. Hu, H. Zhang and Y.Z. Ma (2014). Overexpression of *GmDREB1* improves salt tolerance in transgenic wheat and leaf protein response to high salinity. *Crop Journal*, 2: 120–131.
- Jin, J., F. Tian, D.C. Yang, Y.Q. Meng, L. Kong, J. Luo et al. (2017). Plant TFDB 4.0: toward a central hub for transcription factors and regulatory interactions in plants. *Nucleic Acids Res.*, 45: 1040–1045.
- Kapazoglou, A., M. Gerakari, E. Lazaridi, K. Kleftogianni, E. Sarri, E. Tani, and P.J. Bebeli (2023). Crop wild relatives: A valuable source of tolerance to various abiotic stresses. *Plants*, 12: 328.
- Klucher, K.M., H. Chow, L. Reiser and R.L. Fischer (1996). The *AINTEGUMENTA* gene of *Arabidopsis* required for ovule and female gametophyte development is related to the floral homeotic gene *APETALA2*. *Plant Cell*, 8 (2):137–153.

- Kumar, S., G. Stecher and K. Tamura (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.*, 33: 1870–1874.
- Lam, E., J.S. Jr, J.D. Silva, M. Lawton, S. Bonos, M. Calvino et al. (2009). Improving sugarcane for biofuel engineering for an even better feedstock. *GCB Bioenergy*, 1: 251–255.
- Lata, C., A.K. Mishra, M. Muthamilarasan, V.S. Bonthala, Y. Khan and M. Prasad (2014). Genome-wide investigation and expression profiling of AP2/ERF transcription factor superfamily in foxtail millet (*Setaria italica* L.). *PLoS One*, 9 (11): e113092.
- Li, Z., X. Hua, W. Zhong, Y. Yuan, Y. Wang, Z. Wang, R. Ming and J. Zhang (2020). Genome-wide identification and expression profile analysis of *WRKY* family genes in the Autopolyploid *Saccharum spontaneum*. *Plant Cell Physiol.*, 61(3): 616-630.
- Ling, Q., W. Huang, P. Jarvis (2011). Use of a SPAD-502 meter to measure leaf chlorophyll concentration in *Arabidopsis thaliana*. *Photosynth. Res.*, 107 (2): 209-14.
- Liu, S., X. Wang, H. Wang, H. Xin, X. Yang, J. Yan et al. (2013). Genome-Wide analysis of *ZmDREB* genes and their association with natural variation in drought tolerance at seedling stage of *Zea mays* L. *Plos Genetics*, 9 (9):119–129.
- Moore, P., C. Nagai and M. Fitch (1989). Production and evaluation of sugarcane haploids. *Proc. Int. Soc. Sugar Cane Technol.*, 20: 599–607.
- Nair, S. K., N. Wang, Y. Turuspekov, M. Pourkheirandish, S. Sinsuwongwat, G. Chen et al. (2010). Cleistogamous flowering in barley arises from the suppression of microRNA-guided HvAP2 mRNA cleavage. *Proc. Natl. Acad. Sci. USA*, 107: 490–495.
- Nakano, T., K. Suzuki, T. Fujimura and H. Shinshi (2006). Genome-wide analysis of the *ERF* gene family in *Arabidopsis* and rice. *Plant Physiol.*, 140: 411–432.
- Nakashima, K., Z.K. Shinwari, Y. Sakuma, M. Seki, S. Miura, K. Shinozaki et al. (2000). Organization and expression of two *Arabidopsis DREB2* genes encoding DRE-binding proteins involved in dehydration- and high-salinity-responsive gene expression. *Plant Molecular Biology*, 42 (4): 657–665.
- Roach, B. (1972). Nobilisation of sugarcane. *Proc. Int. Soc. Sugar Cane Technol.*, 1972: 206–216.
- Ross, C.A., Y. Liu and Q.J. Shen (2007). The *WRKY* gene family in rice (*Oryza sativa*). *J. Integr. Plant Biol.*, 49: 827–842.

- Rushton, P.J., I.E. Somssich, P. Ringler and Q.J. Shen (2010). WRKY transcription factors. *Trends Plant Sci.*, 15: 247–258.
- Sakuma, Y., Q. Liu, J.G. Dubouzet, H. Abe, K. Shinozaki and K. Yamaguchi-Shinozaki (2002). DNA-binding specificity of the ERF/AP2 domain of *Arabidopsis* DREBs, transcription factors involved in dehydration- and cold inducible gene expression. *Bioch. Bioph. Res. Co.*, 290: 998–1009.
- Schmidt, R., D. Mieulet, H.M. Hubberten, T. Obata, R. Hoefgen, A.R. Fernie et al. (2013). SALT-RESPONSIVE *ERF1* regulates reactive oxygen species-dependent signaling during the initial response to salt stress in rice. *Plant Cell*, 25 (6): 2115–2131.
- Sharma, M.K., R. Kumar, A.U. Solanke, R. Sharma, A.K. Tyagi and A.K. Sharma (2010). Identification, phylogeny, and transcript profiling of *ERF* family genes during development and abiotic stress treatments in tomato. *Mol. Genet. Genomics*, 284: 455–475.
- Shinozaki, K. and K. Yamaguchi-Shinozaki (1997). Gene expression and signal transduction in water-stress response. *Plant Physiology*, 115 (2): 327.
- Sun, Z.M., M.L. Zhou, X.G. Xiao, Y.X. Tang and Y.M. Wu (2014). Genome-wide analysis of *AP2/ERF* family genes from *Lotus corniculatus* shows *LcERF054* enhances salt tolerance. *Functional and Integrative Genomics*, 14 (3): 453–466.
- Taketa, S., S. Amano, Y. Tsujino, T. Sato, D. Saisho, K. Kakeda et al. (2008). Barley grain with adhering hulls is controlled by an *ERF* family transcription factor gene regulating a lipid biosynthesis pathway. *Proc. Natl. Acad. Sci. USA*, 105 (10): 4062–4067.
- Thamilarasan, S.K., J.I. Park, H.J. Jung and I.S. Nou (2014). Genome-wide analysis of the distribution of *AP2/ERF* transcription factors reveals duplication and *CBFs* genes elucidate their potential function in *Brassica oleracea*. *BMC Genomics*, 15 (12): 71–71.
- Wang, Y., H. Tang, J.D. Debarry, X. Tan, J. Li, X. Wang et al. (2012). *MCScanX*: a toolkit for detection and evolutionary analysis of gene synteny and collinearity. *Nucleic Acids Res.*, 40: e49.
- Wessler, S.R. (2005). Homing into the origin of the AP2 DNA binding domain. *Trends Plant Sci.*, 10: 54–56. Xu, H., K.A. Watanabe, L. Zhang and Q.J. Shen (2016). *WRKY* transcription factor genes in wild rice *Oryza nivara*. *DNA Res.*, 23: 311–323.
- Yan, H.W., L. Hong, Y.Q. Zhou, H.Y. Jiang, S.W. Zhu, J. Fan et al. (2013). A genome-wide analysis of the *ERF* gene family in sorghum. *Genetics and Molecular Research*, 12 (2): 2038–2055.

- Yang, C.Y., F.C. Hsu, J.P. Li, N.N. Wang and M.C. Shih (2011). The AP2/ERF transcription factor *AtERF73/HRE1* modulates ethylene responses during hypoxia in *Arabidopsis*. *Plant Physiology*, 156 (1): 202–212.
- Zahran, M.A., Y.A. El-Amier and R.A. Shawky (2016). Natural vegetation of the Egyptian deserts: Ecology and economic potentialities. *J. Environ. Sci.*, 45 (3-4): 269-282.
- Zhang, G., C. Ming, X. Chen, Z.S. Xu, S. Guan, L.C. Li et al. (2008). Phylogeny, gene structures, and expression patterns of the ERF gene family in soybean (*Glycine max* L.). *J. Exp. Bot.*, 59 (15): 4095–4107.
- Zhang, J., J. Arro, Y. Chen and R. Ming (2013). Haplotype analysis of sucrose synthase gene. *BMC Genomics*, 14: 314.
- Zhang, Q., W. Hu, F. Zhu, L. Wang, Q. Yu, R. Ming and J. Zhang (2016). Structure, phylogeny, allelic haplotypes and expression of sucrose transporter gene families in *Saccharum*. *BMC Genomics*, 17: 88.
- Zhang, J., X. Zhang, H. Tang, Q. Zhang, X. Hua, X. Ma et al. (2018) Allele-defined genome of the autopolyploid sugarcane *Saccharum spontaneum* L. *Nat. Genet.*, 50: 1565–1573.
- Zhao, T. and M.E. Schranz (2017). Network approaches for plant phylogenomic synteny analysis. *Curr. Opin. Plant Biol.*, 36: 129–134.
- Zhu, X.L., L. Qi, X. Liu, S. Cai, H. Xu, R. Huang et al. (2014). The wheat ethylene response factor transcription factor pathogen-induced *ERF1* mediates host responses to both the necrotrophic pathogen *Rhizoctonia cerealis* and freezing stresses. *Plant Physiology*, 164 (3): 1499–1514.

ملاحح التعبير النفاضلي للعائلة الجينية APETALA2/ethylene-responsive factor (AP2/ERF)

في قصب السكر المصري (*Saccharum spontaneum* L.) استجابةً للهرمونات النباتية والإجهاد اللاإحيائي

محمد عويس

وحدة الوراثة والسيتولوجي، قسم الأصول الوراثة، مركز بحوث الصحراء، القاهرة، مصر
قسم البيولوجيا الجزيئية، كلية علوم الحياة، جامعة هاو زينج الزراعية، ووهان، الصين

عائلة الجينات APETALA2/ethylene-responsive factor (AP2/ERF) هي عوامل نسخ خاصة بالنباتات. تلعب AP2/ERFs أدوارًا جوهريّة في تنظيم النمو والتطور، وتحمل الضغوطات الحيوية وغير الحيوية، والاستجابة للهرمونات النباتية. ومع ذلك، لا توجد معلومات عن أدوار العائلة الجينية AP2/ERF في قصب السكر المصري (*Saccharum spontaneum*). في الدراسة الحالية، تم إجراء تحليل على مستوى الجينوم لتحديد جينات AP2/ERF في *S. spontaneum*. باستخدام تقنيات المعلوماتية الحيوية، تم الحصول على إجمالي 288 *SsAP2/ERF*. تم تصنيفهم إلى خمس عائلات فرعية بناءً على دراسة النشوء والتطور، هي AP2 14 و DREB 105 و RAV 4 و ERF 143 و Soloist 1 و 21. تم فحص بنية الجينات ومواقع الكروموسومات والنطاق المحفوظ لجينات *SsAP2/ERF*. على عكس جيناتها المتجانسة، والتي غالبًا ما يتم التعبير عنها في العقد والبراعم، أظهر تحليل التعبير الخاص بالأنسجة أن جينات *SsAP2/ERF* كانت متباينة ويتم التعبير عنها في جميع الأنسجة التي تم فحصها، مع وجود مستويات أعلى قليلاً في الجذور تليها الأوراق والأزهار. ومن المثير للاهتمام، أن الستة جينات *SsAP2/ERF* التي تحتوي على نطاقين AP2 قد أظهرت أنماط تعبير متنوعة استجابة للهرمونات النباتية بما في ذلك حمض الأبسيسيك، وحمض الجبريليك، وحمض الساليسيليك، والميثيل جاسمونات، جنبًا إلى جنب مع العديد من الإجهادات اللاإحيائية مثل الجفاف، وارتفاع الملوحة، وارتفاع درجة الحرارة أو انخفاضها. بشكل موجز، توفر نتائج هذا البحث نظرة عميقة نحو المزيد من الاستكشاف الوظيفي لـ *SsAP2/ERFs* استجابة للهرمونات النباتية والإجهاد اللاإحيائي مع هدف نهائي هو تطوير إنتاج المحاصيل.