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Investigating members of *Arabidopsis* WRKYs transcription factors with differential expression under various stresses using bioinformatics approaches

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Abstract: In the current study, a comprehensive analysis was performed on Arabidopsis WRKY transcription factor (TF) family members that their differential expression (DE) was reported under various stresses in GEO profile database. First, WRKY members with DE were extracted from GEO profile and information of the data set, sequence and their gene structure were obtained. Then, the concept of the intersection of sets was used to select some of WRKY TFs for downstream analysis. DE of candidate members was compared by t-test. The protein– protein interaction network were predicted by STRING web server. A total of 16 *WRKY* genes were identified in the 11 dataset of GEO profile. Analysis of the gene structure showed that 56% of the studied *WRKY* genes have 3 exons and all 16 members are distributed across all five chromosomes of *Arabidopsis*. Also, the results showed that *WRKY40*, *WRKY46*, *WRKY18*, and *WRKY33* were most frequently responsive to various stresses. The protein-protein interaction network showed that WRKY40, WRKY46, WRKY18, and WRKY70 have high interactions with four genes: *MPK4*, *ACS6*, *MKS1*, and *STZ*. Therefore, WRKY40, WRKY46, WRKY18, and WRKY33 can be considered the most important WRKY TFs of *Arabidopsis* at response to various stresses and may find applications in genetic and metabolic engineering projects.

Keywords: GEO profile, exon number, high light, chitooctaose.

Introduction

The concept of stress in plants has been a subject of discussion in scientific communities for more than 8 decades and refers to any adverse conditions and substances that impact or impede the growth, development, and metabolism of plants (Mochizuki et al., 2001; Liu et al., 2016). In plants, due to the nature of their immobility, the definition of stress is completely different from humans and animals. Consequently, plants employ various tolerance and response mechanisms to cope with adverse conditions. Different factors of natural and anthropogenic can be known as stressors and these factors can lead to damage or death of plants depending on the duration and intensity of the stressor. Drought, salt, high and low temperatures, nutrient deficiency, long rainy periods, various insects, and pathogens are some of the known natural stressors. Also, acid rain, various herbicides, and poisons, ozone, increasing UV radiation, and changing the pH of soil and water can be mentioned as stresses with anthropogenic origin (Jäger et al., 2022). In the last three decades, with the development of the concept of plant stress, research technologies have also improved in this field. So that, the number of articles on subject of investigating the response of plants to different types of stress has increased. The response of plants to stress is modulated through deep changes in physiological, biochemical and genomic levels.

In the genomic level, the regulation of gene expression or change of transcriptome is a vital step of the plant's response to stimuli. Recently, high-throughput technologies such as microarray and RNAseq have made it possible to comprehensively investigate the response of plant to stress at the transcriptome level. Transcription factors (TFs) are one of the key factors regulating gene expression. TFs are proteins possessing DNA-binding domains and can change the genes expression and perform the first phase of the genome decoding under different conditions (Lambert et al., 2018; Deng et al., 2022). A significant portion of the plants genome is assigned to TFs, for example in *Arabidopsis* 1771 loci assigned to 2296 TFs, which are divided into 58 families (Jin et al., 2017). Some families of TFs are specifically found in plants and algae, including AP2, NAC, ERF, and WRKY families.

The family of WRKY TFs has a different number of members in various plant species. For example, In *Arabidopsis*, the WRKY TF family with 90 members is one of the most numerous families of TFs (Li et al., 2020). All WRKY proteins have one or two WRKY domains with a length of 60 amino acids, contain a highly conserved amino acid sequence of WRKYGQK, and a zing finger like motif. Based on the type of zing finger-like motif and the number of domains, they are divided into three groups (Jiang et al., 2021). The members of this family regulate the expression of their target genes by binding to W-box, (T)TGAC(C/T), cis-elements in the genes promoter. The members of WRKY family are involved in various processes including growth and development and response to various biotic and abiotic stresses (Jiang et al., 2021; Yu et al., 2023). Overexpression of the *MxWRKY55* gene in transgenic *Arabidopsis* has led to increased resistance to salt stress. Also, the study of transgenic *Arabidopsis* with overexpression of the *GhWRKY33* gene showed this WRKY as a negative regulator mediated the response of plants to drought stress (Wang et al., 2019). Li et al. (2020) reported that the high expression of the *TaWRKY46* gene in *Arabidopsis* led to increased resistance to osmotic stress (Li et al., 2020).

Considering that TFs are one of the important factors in the regulation and coordination of biochemical pathways that are widely used in metabolic engineering (Deng et al., 2022). So that, identification of the most important TFs involved in response to most stress can provide valuable information for genetic and metabolic engineers. Accordingly, in this research, efforts were made to identify and introduce the most important members of the *Arabidopsis* WRKY family of TFs in response to various stresses using information available in databases and through bioinformatics analyses.

Materials and Methods

Dataset extracting

The GEO profile database was searched on the NCBI website (https://www.ncbi.nlm.nih.gov) to identify members of the WRKY TF family with differential expressions. Then, the explanation of the experiment of each of the resulting records was studied through the GEO dataset, the records

related to mutant samples of *Arabidopsis* were removed, and only the samples related to the wild type were included in the analysis. Types of stress were assayed in the present study, which included salinity stress, dehydration stress, highlight, auxin, paraquat, selenate, phosphate deficiency, chitoactase and NEP1. Summary of Information about the dataset studied in this experiment is reported in Table 1.

Table 1. Information of the GEO datasets (expression profiling by array) of assayed in present study.

Dataset name	Dataset ID	No. sample	Stress type	Stress level	Replicates	Time course	PMID	Publication year
Salt stress effect on multiple genotypes: leaf	GSE16765	12	salinity stress	2	2	_	21821598	2011
Gene expression from *Arabidopsis* under high light conditions	GSE22671	9	high light	3	3	_	21531897	2011
Leaf response to paraquat: time course	GSE10464	6	paraquat	2	3	3	14508004	2008
Selenate effect on roots	GSE9311	4	Selenate	2	2	_	18251864	2007
Auxin signaling inhibitors effect on seedlings	GSE1491	21	Auxin	6	3 or 4	_	15466695	2004
Abiotic stress-inducing agents effect on suspension cell cultures	GSE3709	37	a range of abiotic treatments.	17	1 or 3 or 4	_	14730085, 16027974	2005
Whole seedling roots response to salinity stress	GSE7642	12	salinity stress	2	2	5	18436742	2008
Phosphate deficiency effect on roots: time course	GSE25171	24	phosphate deficiency	2	2	4	21248074	2011
Chitin oligomer chitooctaose effect on seedlings	GSE4746	6	chitooctaose (Chitin oligomer)	2	3	_	17722694	2006
Necrosis- and ethylene-inducing peptide effect on dicots	GSE4638	4	Nep1	2	2	_	16698904	2006
mRNA translation and dehydration stress	GSE2268	4	dehydration	2	2	_	15716313	2005

Results and Discussion

In this experiment, eleven datasets were examined, encompassing 139 samples originated from various conditions and organs of the *Arabidopsis* plant. The evaluation of GEO profile database demonstrated that 16 genes of *WRKY* family members showed differential expression under different stimuli. In order to provide better information from each of these members, some nucleotide and protein characteristics are shown in Table 2. The lengths of sequences of *WRKY* members ranged between 810 (*WRKY75*) to 2028 (*WRKY33*) nucleotides. Also, *WRKY75* and *WRKY33* have the minimum and maximum length of the amino acid sequence, respectively.

Table 2. Information of sequence of *Arabidopsis* WRKY members studied in present study.

Gene symbol	Gene ID	Transcript ID	Protein ID	Gene name	Length (nt)	Length (aa)	Chr
WRKY33	818429	NM_129404.4	NP_181381.2	WRKY DNA-binding protein 33	2028	519	2
WRKY40	844423	NM_106732.4	NP_178199.1	WRKY DNA-binding protein 40	1325	302	1
WRKY46	19248	NM_130204.3	NP_182163.1	WRKY DNA-binding protein 46	1354	295	2
WRKY18	829308	NM_119329.4*	NP_567882.1	WRKY DNA-binding protein 18	1740	310	4
WRKY53	828481	NM_118512.3	NP_194112.1	WRKY family transcription factor	1514	324	4
WRKY30	832476	NM_122316.3	NP_568439.1	WRKY DNA-binding protein 30	1290	303	5
WRKY75	831147	NM_121311.5	NP_196812.1	WRKY DNA-binding protein 75	810	145	5
WRKY70	824807	NM_115498.4	NP_191199.1	WRKY DNA-binding protein 70	1470	294	3
WRKY22	827896	NM_116355.3	NP_192034.1	WRKY family transcription factor	1326	298	4
WRKY25	817575	NM_128578.4	NP_180584.1	WRKY DNA-binding protein 25	1776	393	2
WRKY28	827542	NM_117927.3	NP_193551.1	WRKY DNA-binding protein 28	1453	318	4
WRKY48	835012	NM_124329.3	NP_199763.1	WRKY DNA-binding protein 48	1921	399	5
WRKY38	832320	NM_122163.3	NP_197649.2	WRKY DNA-binding protein 38	1306	289	5
WRKY54	818670	NM_129637.3	NP_181607.1	WRKY DNA-binding protein 54	1432	346	2
WRKY26	830601	NM_203017.2*	NP_974746.1	WRKY DNA-binding protein 26	1576	216	5
WRKY45	821270	NM_111063.4	NP_186846.1	WRKY DNA-binding protein 45	1359	147	3

The symbol * indicates that more than one transcript was detected for the desired gene, and here the features of the longest transcript are reported as the canonical transcript

The distribution of the number of exons in the studied members of the WRKY is shown in figure 1. The results showed that 56% (9 members) of the studied members of this family have the exon number of 3. Considering that the amino acid sequence of the members of WRKY family is not very long, the number of exons is predictable. The *WRKY33*, *WRKY18* and *WRKY25* with five exons have the largest number of exons. Also, three genes, *WRKY53*, *WRKY45*, and *WRKY75* have only two exons. Also, the evaluation of the number of transcripts of the studied *WRKY* genes showed that only two genes, *WRKY18* and *WRKY26*, were affected by alternative splicing and produced 3 and 5 transcripts, respectively. The map and chromosomal distribution of the WRKY members revealed that the studied genes are scattered in all chromosomes and chromosome 5, with 5 genes, showed the highest density, and chromosome 1 showed the lowest density (Figure. 2).

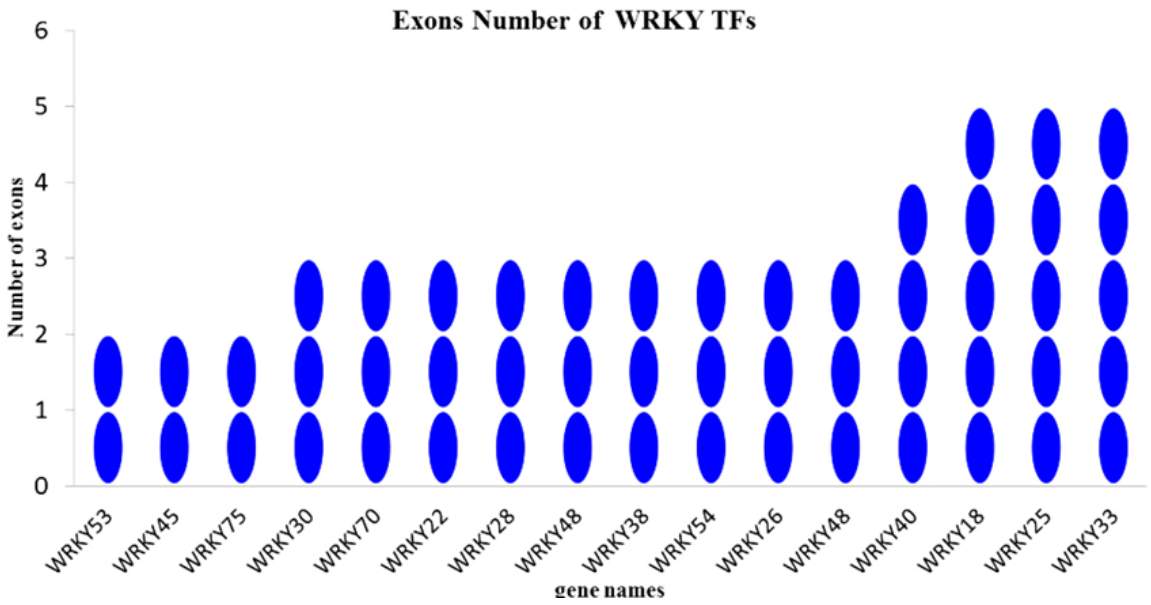

Figure 1. The exons number of the *Arabidopsis WRKY* members studied in present research.

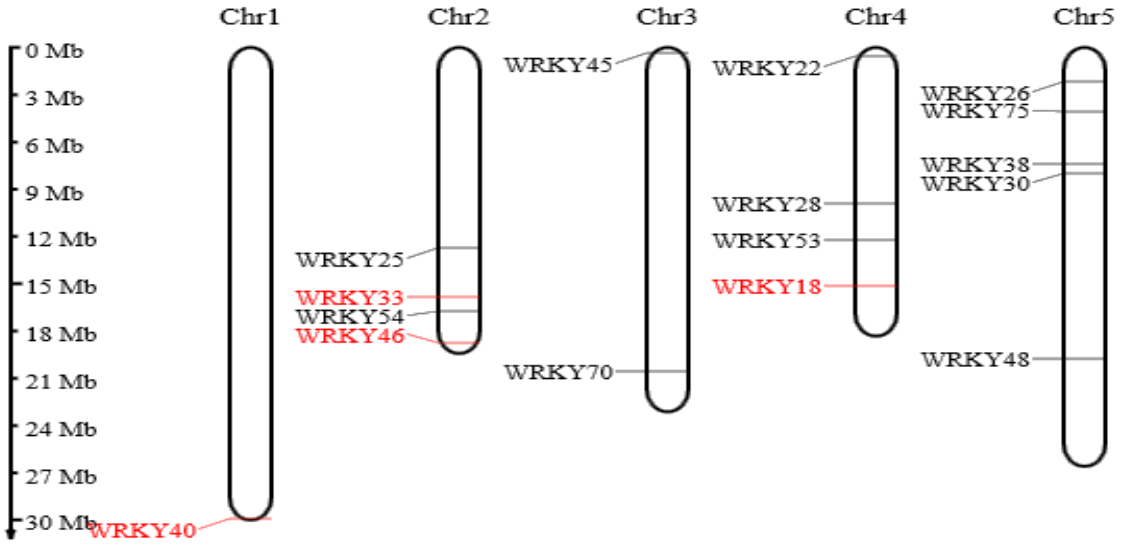

Figure 2. Distribution the *Arabidopsis WRKY* members studied in present research on 5 chromosomes of *Arabidopsis* . Four candidate members for further investigation are marked in red font.

The evaluation of the frequency of WRKY members in the dataset revealed that four genes of *WRKY40*, *WRKY46*, *WRKY18* and *WRKY33* showed differential expression in more than 50% of the studied datasets, such that *WRKY40*, *WRKY33*, and *WRKY46* have been observed in 8, 7 and 6 studies, respectively (Figure. 3). On the other hand, more detailed investigations revealed that all four mentioned members are present in three datasets of GSE4746, GSE9311 and GSE22671. Therefore, in the

following we focused on the investigation of *WRKY40*, *WRKY46*, *WRKY18* and *WRKY33* genes in three datasets of GSE4746, GSE9311 and GSE22671. The expression values of these four genes in the three datasets were reported in the Figure 4.

In the GSE9311 dataset, the effect of selenate (Se) on the expression of genes in the root was evaluated. Se and sulfate are chemical analogs and can be taken up by plants through the same transporters and enzymes. Unlike many other organisms, Se is not essential for higher plants. In plants, its excessive amount is toxic and limits growth. Both Se deficiency and toxicity doses are dangers worldwide (Van Hoewyk et al., 2008). The results of the expression of four *WRKY* in this dataset showed that the expression of all four studied genes increased significantly, so that *WRKY40* showed the highest expression value equal to 5627 and 95 FPKM in control and treatment samples, respectively. Also, the *WRKY46* gene was showed the lowest expression value with means 75 and 1975 in control and treatment samples, respectively (Figure. 4a).

In the GSE4746 dataset, the changes of *Arabidopsis* transcriptome were evaluated by chitectase treatment. Chitectase is an oligomer of chitin that is found in the cell wall of fungi, insects and nematodes and is used as a fungal elicitor in researches (Libault et al., 2007).

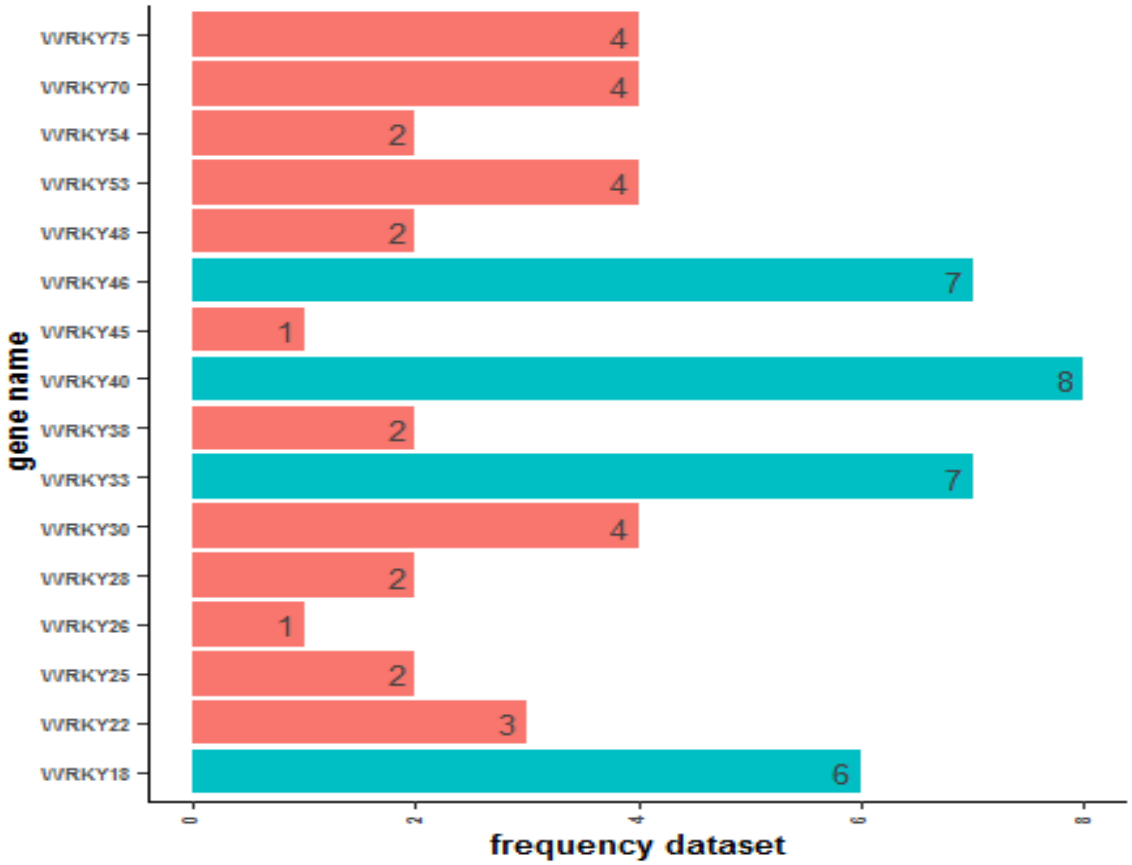

Figure 3. The number of datasets that reported the differential expression of each *Arabidopsis WRKY* member in present study. The genes that present in more than 50% of the data sets were marked with blue columns.

The results of investigations of this dataset showed that the expression of all four candidate genes increased significantly, so that *WRKY33* showed the highest expression value (mean FPKM = 3588) and *WRKY46* showed the lowest value (mean FPKM =1727). Also, in the control (water) samples, the mean expression of these two genes was estimated 359 and 118, respectively (Figure 4b).

The evaluation of differential expression in the GSE22671 data set is shown in the Figure 4c. In this dataset, cell culture suspension, that has active chloroplast cells, was exposed to high light and its defense response was investigated against high light stress. The results showed that the expression of *WRKY33*, *WRKY40* and *WRKY43* genes increased significantly in response to high light, but our statistical analysis did not confirm the significant increase in expression of *WRKY18* gene.

When plants sense stress, the signals of stress perception are activated and transformed into cell. Inside the cells, plants recruit a complex signaling transduction network for trigger chemical and molecular responses.

The change of balance of reactive oxygen species (ROS) and the concentration of calcium play the role of an intercellular second messenger, as well as the activation of kinases cascade pathway such as MAPK (mitogen activated protein kinase). Subsequently, the MAPK cascade pathway activates transcription factors as cis-regulatory elements of responsive genes. Among the TFs, the expression of *WRKY* TFs changes rapidly in response to various stresses (Gonzalez-Perez et al., 2011). Recently, the WRKY TFs were introduced as negative and positive regulators in response to various stresses in plant species. Zhang et al. (2006) reported that WRKY33 regulates the antagonistic interaction between defense pathways mediating responses to necrotrophic pathogens and *P. syringae* (Zheng et al., 2006). In addition, the role of WRKY33 as a negative regulator in oxidative and abscisic acid (ABA) stresses has been confirmed in *Arabidopsis*. Also, the results showed that AtWRKY18, AtWRKY40 and AtWRKY60, control resistance to pathogens by forming homologous or heterologous dimers (34). It has also been reported that WRKY40 is involved as a negative regulator of ABA during the stages of seed germination and post-germination growth (Geilen and Bohmer, 2015).

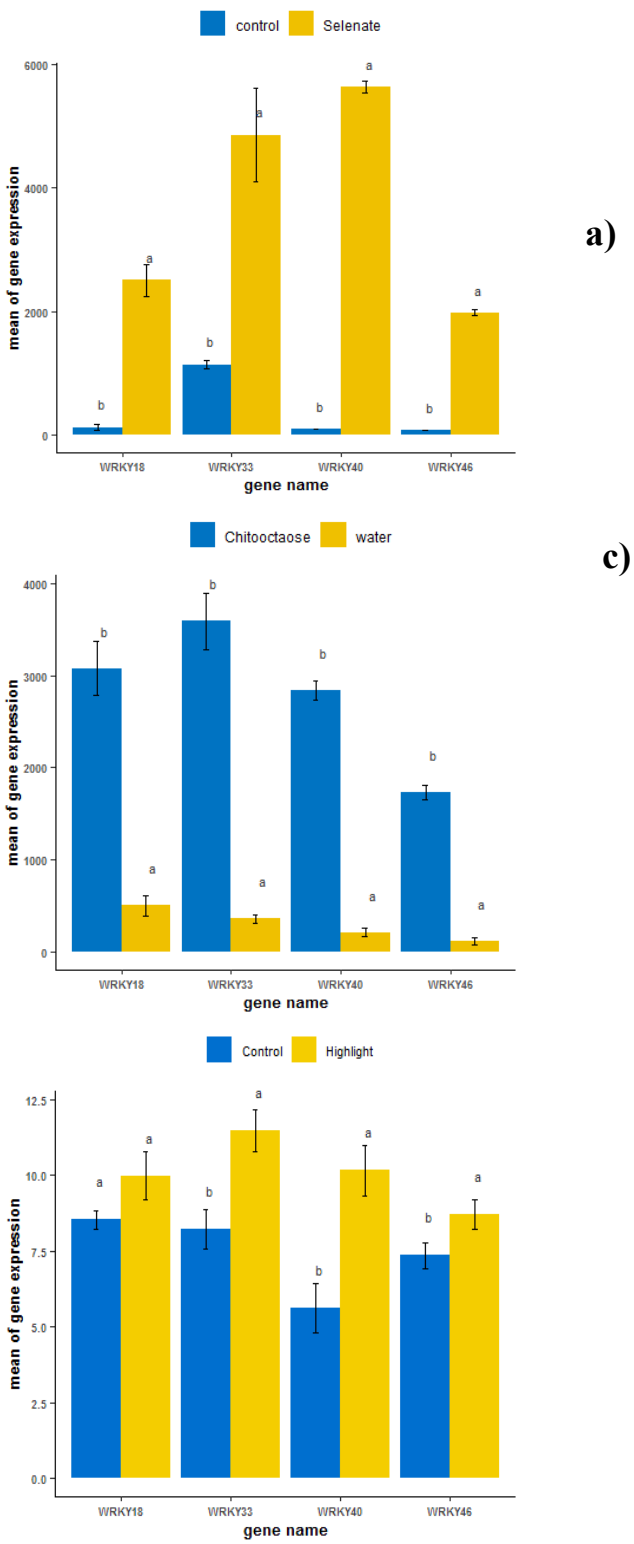

Figure 4. The differential expression of the *Arabidopsis* WRKY members selected in present study. a) GSE9311 dataset (selenate treatment) b) GSE4746 dataset (chitectase treatment) c) GSE22671 dataset (high light treatment).

More studies also showed that other members of the WRKY family of transcription factors in *Arabidopsis* are affected by various stresses. For example, WRKY26, WRKY25, and WRKY39 have been introduced as transcription factors involved in heat stress and WRKY34 as a negative regulator of cold resistance in *Arabidopsis* (Zheng et al., 2006; Li et al., 2011).

The number of the WRKY family members is different in various species, so the concept of orthology and orthologous genes in this family is very broad. Until now, the effective role of the WRKY members in the response to various stresses has been reported in different species. It has been reported that WRKY25, WRKY63 and WRKY21 are involved in the tolerance of salt and drought stresses (Jia et al., 2015; Liu et al., 2016; Wang et al., 2021). In rice, the WRKY74 and WRKY76 have been introduced as key factors in response to cold stress, and WRKY89 is a TF involved in UV light resistance (Wang et al., 2007; Yokotani et al., 2013; Dai et al., 2016).

The interactions between WRKYs members and between WRKYs with other proteins regulate the various pathways involved in response to stress. Analysis of protein-protein interaction network showed that among the four candidate proteins, WRKY40, WRKY18, and WRKY33, have a very strong interaction with each other and with another group of proteins. Another member of WRKY family, WRKY70, was also present in the predicted protein network (Figure 5). The WRKY70 showed differential expression in only two of the studied datasets in current study (Figure 3).

Also, the protein network formed two distinct group of protein-protein network. The GUN5 protein plays role of connected node for formed two groups (Figure 5). The GUN5 protein is a multifunctional protein that plays important role in the production of chlorophyll, a photosynthetic pigment, by catalyzing the metabolism of porphyrin-containing components. Also, due to the function of magnesium clathase, this enzyme plays an important role in the plastid-to-nucleus signaling pathway, by coordinating the genes involved in photosynthesis with the two origins of the chloroplast and the nucleus. In addition, the study of ABA sensitive phenotypes showed this gene is a positive regulator of the signaling pathway of ABA hormone in stomatal guard cells during seed germination (Mochizuki et al., 2001).

The protein- protein interaction network also showed that the four genes of MPK4, ACS6, MKS1 and STZ interact with all four WRKY members that are present in the predicted protein network. The thickness of the edges between these nods confirms interactions with very strong data support (Figure 5). Studies have reported the role of each of these proteins in response to various types of stress. The MAPK signal transduction cascade is utilized by eukaryotic cells to transduce a wide variety of extracellular signals such as growth factors, hormones, and stress stimuli (Li et al., 2015).

The results showed that mapk4 is one of the key members of the signaling pathway in *Arabidopsis* and regulating the expression of genes responding to various biotic and abiotic stresses and also plays an important role as a negative regulator in pathogen defense (Li et al., 2015).

Also, Liu and Zhang (2004) showed that MAPK6 enzyme leads to the stimulation of ethylene production in response to various stresses by phosphorylation of the ACS6 enzyme. The enzyme ACS6 which converts S-adenosyl-L-methionine (SAM) to 1-aminocyclopropene 1-carboxylase (ACC) and is involved in the production of ethylene is stimulated by bacterial flagellin. The ACC is considered as a central precursor of ethylene, and the increase in ethylene production in response to a variety of biological stresses have been confirmed in plants (Chen et al., 2020). It has also been reported that the activity of ACS6 enzyme leads to an increase in stomatal density of the epidermis of *Arabidopsis* leaves in response to drought stress (Jia et al., 2021).

Another protein present in protein- protein interaction network is MKS1, which is a defense response regulator in plants, and acts as a downstream substrate of the MAPK4 enzyme in the salicylic acid-dependent pathway. Its interaction with the transcription factors of WRKY33 and WRKY25 has been proven, and may act as a mediator between MAPK and downstream transcription factors (Andreasson et al., 2005).

#node	Gene name
ACS6	1-aminocyclopropane-1-carboxylic acid (acc) synthase 6
ALB1	Magnesium-chelatase subunit ChlD, chloroplastic
AT1G74470	Geranylgeranyl diphosphate/geranylgeranyl-bacteriochlorophyllide a reductase
BHLH92	Basic helix-loop-helix (bHLH) DNA-binding superfamily protein
CHLI1	P-loop containing nucleoside triphosphate hydrolases superfamily protein
CHLI2	Magnesium-chelatase subunit ChlI-2, chloroplastic
CHLM	Magnesium protoporphyrin ix methyltransferase, chloroplastic
CML38	Calcium-binding protein CML38
ERF11	Ethylene-responsive transcription factor 11
GUN4	Tetrapyrrole-binding protein, chloroplastic
GUN5	Magnesium-chelatase subunit chlh, chloroplast
HEMG2	Protoporphyrinogen oxidase 2, chloroplastic/mitochondrial
MKS1	MAP kinase substrate 1
MPK4	Mitogen-activated protein kinase 4
PPOP1	Protoporphyrinogen/coproporphyrinogen iii oxidase
RHL41	C2H2-type zinc finger family protein
STZ	Related to Cys2/His2-type zinc-finger proteins found in higher plants.
WRKY18	Arabidopsis thaliana wrky dna-binding protein 18
WRKY33	Probable WRKY transcription factor 33
WRKY40	Probable WRKY transcription factor 40
WRKY70	Arabidopsis thaliana wrky dna-binding protein 70

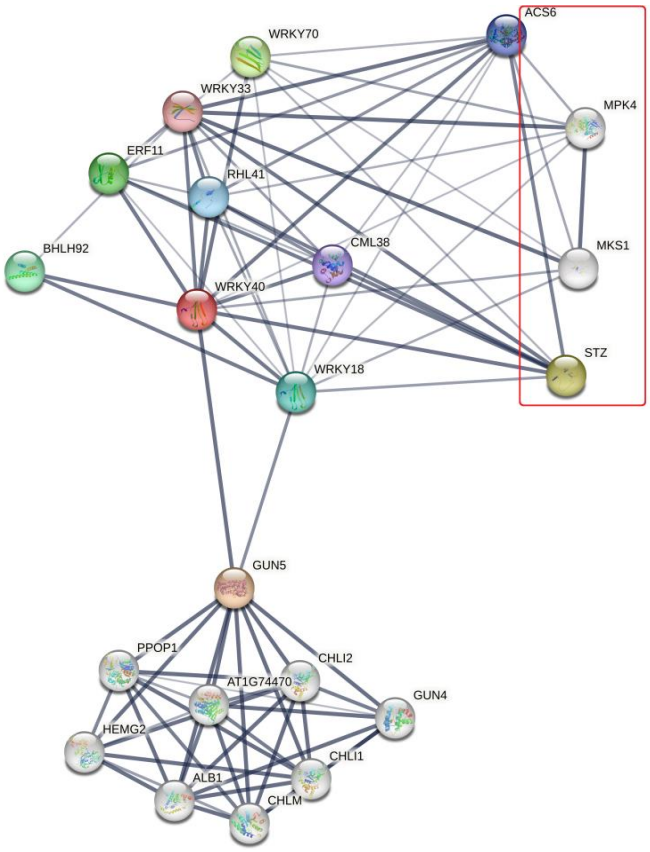

Figure 5. The protein-protein interaction network with present WRKY40, WRKY18, WRKY33 and WRKY70. The confidence level of interactions was considered more than 0.7 (high level). Full names of nodes defined in table.

Its interaction with the transcription factors of WRKY33 and WRKY25 has been proven, and may act as a mediator between MAPK and downstream transcription factors (1). STZ protein is known as a transcription repressor in abiotic stresses. It controls the expression of LOX3 (jasmonic acid biocenter gene) and *JAZ1* genes (key repressor in jasmonic acid cascade signaling pathway) (Mittler et al., 2006; Xie et al., 2012). In transgenic *Arabidopsis* plants, overexpressing STZ1 growth retardation has been observed to increase tolerance to drought, salt, heat and osmotic stresses (Sakamoto et al., 2004).

Conclusion

The findings of this study reveal 16 *Arabidopsis WRKY* genes exhibiting differential expression in response to diverse stress conditions. Subsequent bioinformatics-based analyses such as expression analysis and the examination protein- protein interaction network of WRKY proteins revealed that four members of WRKY40, WRKY46, WRKY18 and WRKY33 are the most crucial within this family. These members can be used in genetic and metabolic engineering projects aimed at developing stress-tolerant plants.

Supplementary Materials

No supplementary material is available for this article.

Author Contributions

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Conflicts of Interest

The author declares no conflict of interest.

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مقاله پژوهشی

بررسی اعضای فاکتورهای رونویسی WRKYs عربیدوپسیس با بیان افتراقی تحت تنش‌های مختلف با استفاده از رویکردهای بیوانفورماتیک

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چکیده: در مطالعه حاضر، تجزیه و تحلیل بر روی اعضای خانواده فاکتورهای رونویسی WRKY در گیاه عربیدوپسیس که بیان افتراقی آنها (DE) تحت تنش‌های مختلف در پایگاه داده GEO profile گزارش شده بود، انجام شد. ابتدا اعضای *WRKY* همراه با DE از GEOprofile اخذ و اطلاعات مجموعه داده‌ها، توالی و ساختار ژنی آنها به دست آمد. سپس از مفهوم اشتراک مجموعه‌ها برای انتخاب برخی از WRKYها برای تحلیل پایین دستی استفاده شد. بیان افتراقی اعضای انتخاب شده با آزمون t مقایسه و شبکه برهمکنش پروتئین – پروتئین توسط وب سرور STRING پیش‌بینی شد. در مجموع ۱۶ ژن *WRKY* در ۱۱ مجموعه داده GEO profile شناسایی شد. تجزیه و تحلیل ساختار ژن نشان داد که ۵۶ درصد از ژن‌های *WRKY* مورد مطالعه دارای ۳اگزون هستند و هر ۱۶ عضو در هر پنج کروموزوم عربیدوپسیس توزیع شده‌اند. همچنین نتایج نشان داد که *WRKY40*، *WRKY46*، *WRKY18* و *WRKY33* بیشترین فراوانی را در پاسخ به تنش‌های مختلف داشتند. شبکه برهمکنش پروتئین–پروتئین نشان داد که WRKY40، WRKY46، WRKY18 و WRKY70 برهمکنش بالایی با چهار ژن *MPK4*، *ACS6*، *MKS1* و *STZ* دارند. بنابراین، WRKY40، WRKY46، WRKY18 و WRKY33 می‌توانند مهم‌ترین TFهای WRKY عربیدوپسیس در پاسخ به تنش‌های مختلف باشند و در پروژه‌های مهندسی ژنتیک و متابولیک مورد استفاده قرار گیرند.

کلمات کلیدی: GEO profile، تعداد اگزون، نور زیاد، کیتوکتاز.

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