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Characterization of *WRKY* Gene in Cereal

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ABSTRACT

The study examines the *WRKY* gene in cereal crops, which plays a crucial role in responding to both abiotic and biotic stresses. Understanding this gene offers valuable insights into the function of the WRKY transcription factor in regulating the expression of genes involved in stress responses. The research aims to analyze *WRKY* genes in cereal plants, identifying motifs and domains related to their response to these stresses. For this purpose, 26 *WRKY* gene sequences from rice (*Oryza sativa* subsp. japonica), soybeans (*Glycine max*), sorghum (*Sorghum bicolor*) and corn (*Zea mays*) were analyzed. Protein sequences were obtained from NCBI and processed using various bioinformatics tools. Phylogenetic analysis was conducted using Mega11, sequence alignment with ClustalW, motif analysis with MEME and domain analysis with InterProScan. The phylogenetic analysis results indicate that the *WRKY* genes are grouped by plant species rather than isoenzymes. Sequence alignment further supports this species-based grouping. Motif analysis using MEME identified 10 motifs in the 26 *WRKY* gene sequences associated with both abiotic and biotic stresses. The WRKYGQK motif was present in all *WRKY* genes, with the PEDGYQWRKYGQKVIKGNPYPRAYYRCTM motif emerging as the dominant motif across all cereal sequences. In this study, five cereal sequences were classified into Group I, fifteen into Group II, four into Group III and two sequences remained unclassified. The findings suggest that conserved motifs, such as WRKYGQK, may serve as active sites necessary for WRKY transcription factors to bind to target gene promoters and regulate their expression in response to stresses. This discovery enhances our understanding of the role of these transcription factors in controlling genes involved in stress responses. The presence of WRKY and zinc finger motifs in most WRKY transcription factor sequences appears to contribute to clustering genes in the phylogenetic tree by genus rather than isoform type. This study demonstrates that *WRKY* genes can be used for stress tolerance screening and improving plant stress tolerance through transgenic technology or breeding.

1. Introduction

Strategies have been developed to build tolerance and adaptation to abiotic and biotic stresses in plants, as plants are unable to escape from predators or environmental changes. Abiotic stresses, such as drought, cold weather, physical injury and salinity, as well as biotic stresses, such as fungal, bacterial and viral attacks, are detected through complex signal transduction networks that result in

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physiological, biochemical and gene expression changes in plants [1-4]. A family of transcription factors, central to this process, has evolved uniquely in plants to coordinate gene expression. The *WRKY* protein class, previously studied by Eulgem *et al.*, (2000) and Ulker & Somssich (2004) [5,6], like ethylene-responsive factors (ERF) [7], DNA-binding domain proteins (Dof) [8] and basic leucine zipper (bZIP) domain proteins [9] is a protein family with numerous members that regulate stress responses in plants. *WRKY* genes were identified over twenty years ago as *SPF1* in sweet potato (*Ipomoea batatas* L.) [10]. These genes possess a highly conserved WRKY domain, comprising 60 amino acids. The family is named WRKY due to the presence of this motif. WRKY transcription factors are classified into three groups based on the number of WRKYGQK domains and zinc finger motifs: Group I has two domains, while Groups II and III have one domain. Groups I and II possess a C2H2-type zinc finger motif, whereas Group III has a C2HC zinc finger motif. Group II members are further classified into subgroups IIa to IIe based on additional short, conserved structural motifs [8].

WRKY transcription factors are involved in various plant biological processes, including root hair development [11], seed size [12,13], pollen development [14], growth [15], flowering [15], fruit ripening [16] and leaf senescence [16,17]. WRKY transcription factors modulate plant hormone signaling pathways [18]. Despite playing a significant role in plant growth, development and signaling pathways, the most significant function of WRKY proteins that have been extensively studied and widely reported is the transcriptional regulation of responses to abiotic and biotic stresses. In *Arabidopsis thaliana*, *AtABO3*, one of the WRKY proteins, is involved in the plants' response to drought stress [19], while Os*WRKY*11 in rice is induced by heat stress and enhances tolerance to high temperatures [20].

In the study of genetic architecture, transcription activation and silencing are key areas of focus. WRKY transcription factors also play a crucial role in biotic stress (fungal or bacterial pathogens). Several WRKY TFs have been shown to confer resistance to biotic stress imposed by fungal or bacterial pathogens by influencing related genes [21]. The function and role of WRKY proteins in plant immune responses are remarkable. For example, Xu *et al.,* (2006) [22] found that AtWRKY18*, AtWRKY40* and *AtWRKY60* interact with each other and play different roles in the plants' response to two types of pathogens - *Pseudomonas syringae* and *Botrytis cinerea*. Molecular complementation and gene silencing have confirmed that *WRKY33* homologs in Arabidopsis and tomato (*S. lycopersicum* L.) play a critical role in resistance to *B. cinerea* [23]. In the study of the BROWN PLANTHOPPER RESISTANCE 14 (*BPH14*) gene, it was reported that this gene mediates insect resistance through interactions with *WRKY*46 and *WRKY*72, which can bind to receptor-like cytoplasmic kinase genes and callose synthase genes in rice. These findings shed light on the role of *WRKY* in resistance to insect pests. Overall, it is evident that WRKY proteins can mediate plant defense mechanisms in various ways.

Although the WRKY family plays an essential role in various plant biological processes, understanding how these genes regulate these processes has not been thoroughly studied [24]. In wheat, the majority (8 out of 15) TaWRKY genes are transcribed in response to cold, heat, salt and PEG treatments [25]. To the best of our knowledge, only a few *WRKY* genes have been reported in maize. ZmWRKY17 can regulate the transcription of several stress-related and ABA-related genes, ultimately increasing salt stress resistance and reducing ABA sensitivity [26]. ZmWRKY33 can be activated by several abiotic stresses such as high salt, dehydration, cold and ABA treatment, and it enhances salt stress tolerance in transgenic Arabidopsis [27]. These studies demonstrate the role of ZmWRKY in enhancing resistance to abiotic stress. Although more than 100 members of the *WRKY* gene family in maize have been found, the expression pattern of ZmWRKY in various maize tissues under abiotic stress has not been investigated at the genome level [28]. Many details regarding the *WRKY* gene family in maize still need to be further elucidated [29].

The role of WRKY transcription factors in abiotic stress response and growth and development in soybean (Glycine max) has been identified. GmWRKY21 or GmWRKY54 expressed in Arabidopsis thaliana enhances tolerance to cold weather, salt stress and drought [30]. Related WRKY proteins, GmWRP1, are important in legume symbiosis, growth and development [31]. However, little has been characterized in the response of these genes to biotic stress. It has been reported that GmWRKY31 enhances resistance to *Phytophthora sojae*, while the loss of this gene increases disease susceptibility [32]. Sorghum is the fifth most important cereal crop in terms of production and field usage. *SbRD19, a sorghum gene that responds to drought stress, is regulated by SbWRKY30, which also increases plant tolerance to drought* [33,34]. These studies confirm that WRKY transcription factors play an important role in development and suggest the potential for their use in enhancing cereal crop resistance to abiotic and biotic stresses.

The purpose of this study is to examine the similarities or differences among *WRKY* genes in cereals and to identify the motifs or domains present in cereal *WRKY* gene sequences through bioinformatics analysis and associate the presence of specific motifs/domains with gene function in either abiotic or biotic stress.

2. Methodology

2.1 Characterization of WRKY Genes in Cereals

To characterize *WRKY* proteins in cereals, several bioinformatics tools were employed to ensure a comprehensive analysis. First, the *WRKY* protein sequences of cereals were downloaded from the NCBI database (http://www.ncbi.nlm.nih.gov/). The process of characterizing rice was similar to other species, using the Hidden Markov Model (HMM) for BLASTP (Basic Local Alignment Search Tool for Proteins). The Hidden Markov Model was utilized to identify potential WRKY transcription factor candidates within the downloaded protein sequences, while BLASTP was the software used to compare the protein sequences against the protein database. MEGA 11 (http://www.megasoftware.net/mega.php) was used for manual inspection to remove erroneous candidate sequences and overlapping or redundant information, ensuring the accuracy of the selected WRKY protein candidates.

2.2 Sequence Alignment Analysis

This analysis was conducted using ClustalW in the MEGA 11 software. A total of twenty-six (26) sequences in FASTA format were used. The parameters for pairwise alignment included setting the "gap opening penalty" to 10 and the "gap extension penalty" to 0.10, while for the multiple alignment, the "gap opening penalty" was set to 10 and the "gap extension penalty" to 0.20.

2.3 Domain Analysis

InterProScan (https://www.ebi.ac.uk/interpro/) was used to predict and annotate protein domains in *WRKY* genes. InterProScan scans protein sequences against various databases, identifying conserved domains, functional sites and important motifs associated with the WRKY transcription factor family. This aids in understanding the characteristics, structure and function of *WRKY* genes.

2.4 Motif Analysis

Twenty-six (26) *WRKY* gene sequences in FASTA format were input into the Motif Elicitation (MEME) software (https://meme-suite.org/meme/tools/meme) to predict motifs within the *WRKY* genes. Each input sequence included a description line starting with the symbol “>” followed by the sequence ID and the following line with the *WRKY* gene sequence. The parameter for the number of predicted motifs was set to 10, and the motif distribution was set with "zero or one occurrence per sequence (zoops)’. This helps identify motifs that appear in each sequence, providing deep insights into the conservation and function of these motifs within the *WRKY* gene family.

2.5 Phylogenetic Analysis Based on WRKY Domain

A phylogenetic tree was constructed using the WRKY domain sequences to determine the relationships among WRKY proteins in rice and other cereals. This analysis was conducted with MEGA 11 (http://www.megasoftware.net/mega.php) using the Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods, with RAxML and PhyML employed for better classification. Several parameters were set to ensure the accuracy of the resulting phylogenetic tree. For NJ, the phylogenetic test was set to 1000 bootstrap replicates. The Poisson model was selected, the rate among sites was uniform and the treatment of gaps or missing data was pairwise deletion. For ML, the phylogenetic test used was 1000 bootstrap replicates, the model or method selected was the Jones-Taylor Thornton (JTT) model, the rate among sites was set to uniform and the treatment of gaps or missing data was partial deletion.

3. Results

3.1 Introduction

To understand the architecture of *WRKY* genes and how motifs, classification and phylogeny explain the functions and relationships among these genes in cereals, several analyses were conducted. This research aimed to characterize WRKY proteins in 26 cereal sequences, perform sequence alignment analysis, identify motifs within *WRKY* genes, predict and determine the positioning of protein domains and conduct phylogenetic analysis based on the WRKY domain. All of these steps are essential to provide deeper insights into the conservation, structure and function of the *WRKY* gene family across various cereal species.

3.2 Sequence Alignment Analysis of WRKY Genes

The sequence alignment of 26 *WRKY* genes was observed and analyzed using ClustalW in the Mega11 software. Conserved peptide regions carrying the WRKYGQK sequence were found in all *WRKY* genes and this sequence is highlighted in a box in Figure 1. This alignment shows that this motif is highly conserved among all *WRKY* proteins from the selected cereal plants.

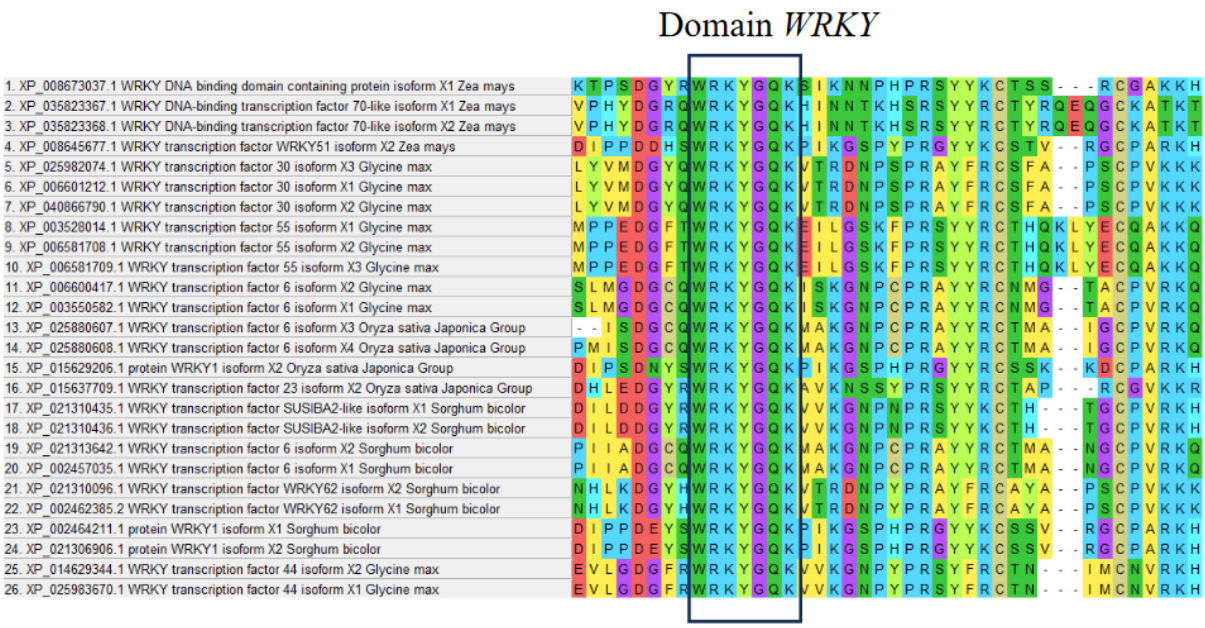

Fig. 1. Sequence alignment indicates presence of the WRKY motif/domain as indicated by a black box

3.3 Domain and Motif Analysis of WRKY Genes in Cereals

Table 1 shows that all cereal sequences possess the WRKY domain and zinc finger motif, except for WRKY transcription factor 62 isoforms X1 and X2 (*S. bicolor*), which lack the zinc finger motif. Most sequences have a single WRKY domain, except for WRKY51 isoform X2 (*Z. mays*), WRKY SUSIBA2-like isoforms X1 and X2 (*S. bicolor*), and WRKY44 isoforms X1 and X2 (*Glycine max*), which contains two WRKY domains. Sequences with two WRKY domains are classified into Group I. Fifteen sequences belong to Group II, and four are in Group III, including WRKY55 isoforms X1, X2 and X3 (*G. max*) and WRKY1 isoform X2 (*O. sativa japonica* Group). WRKY62 isoforms X1 and X2 (*S. bicolor*) are unclassified. WRKY6 (*S. bicolor*) is placed in subgroup IIb, while *WRKY1* (*S. bicolor*) is classified into IId. Additionally, WRKY1 protein isoforms X1 and X2 (*S. bicolor*) and WRKY1 protein isoform X2 (*O. sativa japonica* Group) feature the zinc cluster domain (Table 2).

Table 1
Number of domain WRKY and group of cereals

Sequence name	Number of domain *WRKY*	Group
WRKY DNA binding domain-containing protein isoform X1 [*Zea mays*]	1	II
WRKY DNA-binding transcription factor 70-like isoform X1 [*Zea mays*]	1	II
WRKY DNA-binding transcription factor 70-like isoform X2 [*Zea mays*]	1	II
WRKY transcription factor *WRKY51* isoform X2 [*Zea mays*]	2	I
WRKY transcription factor 30 isoform X3 [*Glycine max*]	1	II
WRKY transcription factor 30 isoform X1 [*Glycine max*]	1	II
WRKY transcription factor 30 isoform X2 [*Glycine max*]	1	II
WRKY transcription factor 55 isoform X1 [*Glycine max*]	1	III
WRKY transcription factor 55 isoform X2 [*Glycine max*]	1	III
WRKY transcription factor 55 isoform X3 [*Glycine max*]	1	III
WRKY transcription factor 6 isoform X2 [*Glycine max*]	1	II
WRKY transcription factor 6 isoform X1 [*Glycine max*]	1	II
WRKY transcription factor 6 isoform X3 [*Oryza sativa japonica* Group]	1	II
WRKY transcription factor 6 isoform X4 [*Oryza sativa japonica* Group]	1	II
protein *WRKY1* isoform X2 [*Oryza sativa japonica* Group]	1	III
WRKY transcription factor 23 isoform X2 [*Oryza sativa japonica* Group]	1	II

WRKY transcription factor SUSIBA2-like isoform X1 [*Sorghum bicolor*]	2	I
WRKY transcription factor SUSIBA2-like isoform X2 [*Sorghum bicolor*]	2	I
WRKY transcription factor 6 isoform X2 [*Sorghum bicolor*]	1	IIb
WRKY transcription factor 6 isoform X1 [*Sorghum bicolor]*	1	IIb
WRKY transcription factor *WRKY62* isoform X2 [*Sorghum bicolor*]	1	-
WRKY transcription factor *WRKY62* isoform X1 [*Sorghum bicolor*]	1	-
protein *WRKY1* isoform X1 [*Sorghum bicolor*]	1	IId
protein *WRKY1* isoform X2 [*Sorghum bicolor*]	1	IId
WRKY transcription factor 44 isoform X2 [*Glycine max*]	2	I
WRKY transcription factor 44 isoform X1 [*Glycine max*]	2	I

Sequence	Sequence Name	Homolog Superfamily (IPR036576)	Domain *WRKY* (IPR003657)	Domain Cluster Zinc (IPR018872)	Length of Amino Acid
XP_008673037.1	*WRKY* DNA binding domain-containing protein isoform X1 [*Zea mays*]	143-215	155-215		352
XP_035823367.1	*WRKY* DNA-binding transcription factor 70-like isoform X1 [*Zea mays*]	155-238	165-238		346
XP_035823368.1	*WRKY* DNA-binding transcription factor 70-like isoform X2 [*Zea mays*]	154-237	164-237		345
XP_008645677.1	*WRKY* transcription factor *WRKY51* isoform X2 [*Zea mays*]	222-309	242-308	197-245	325
XP_025982074.1	*WRKY* transcription factor 30 isoform X3 [*Glycine max*]	120-187	130-190		195
XP_006601212.1	*WRKY* transcription factor 30 isoform X1 [*Glycine max*]	119-217	130-218		312
XP_040866790.1	*WRKY* transcription factor 30 isoform X2 [*Glycine max*]	119-195	130-196		290
XP_003528014.1	*WRKY* transcription factor 55 isoform X1 [*Glycine max*]	163-236	173-236		331
XP_006581708.1	*WRKY* transcription factor 55 isoform X2 [*Glycine max*]	162-235	172-235		330
XP_006581709.1	*WRKY* transcription factor 55 isoform X3 [*Glycine max*]	161-234	171-234		329
XP_006600417.1	*WRKY* transcription factor 6 isoform X2 [*Glycine max*]	165-243	176-242		390
XP_003550582.1	*WRKY* transcription factor 6 isoform X1 [*Glycine max*]	165-244	177-243		391
XP_025880607.1	*WRKY* transcription factor 6 isoform X3 [*Oryza sativa japonica* Group]	191-265	198-264		491
XP_025880608.1	*WRKY* transcription factor 6 isoform X4 [*Oryza sativa japonica* Group]	300-377	310-376		458
XP_015629206.1	protein *WRKY1* isoform X2 [*Oryza sativa japonica* Group]	310-388	321-387	288-324	402
XP_015637709.1	*WRKY* transcription factor 23 isoform X2 [*Oryza sativa japonica* Group]	173-249	183-248		338
XP_021310435.1	*WRKY* transcription factor SUSIBA2-like isoform X1 [*Sorghum bicolor*]	213-289 390-463	224-288 398-463		611
XP_021310436.1	*WRKY* transcription factor SUSIBA2-like isoform X2 [*Sorghum bicolor*]	124-200, 301-374	135-199, 309-374		522
XP_021313642.1	*WRKY* transcription factor 6 isoform X2 [*Sorghum bicolor*]	269-346	279-345		559
XP_002457035.1	*WRKY* transcription factor 6 isoform X1 [*Sorghum bicolor*]	269-346	279-345		570
XP_021310096.1	*WRKY* transcription factor *WRKY62* isoform X2 [*Sorghum bicolor*]	104-178	112-178		294
XP_002462385.2	*WRKY* transcription factor *WRKY62* isoform X1 [*Sorghum bicolor*]	105-179	113-179		295
XP_002464211.1	protein *WRKY1* isoform X1 [*Sorghum bicolor*]	269-346	279-345	236-282	352
XP_021306906.1	protein *WRKY1* isoform X2 [*Sorghum bicolor*]	268-345	278-344	236-281	351
XP_014629344.1	*WRKY* transcription factor 44 isoform X2 [*Glycine max*]	154-230 339-414	170-229 348-413		435
XP_025983670.1	*WRKY* transcription factor 44 isoform X1 [*Glycine max*]	352-427	361-426		448

Fig. 2. Domain WRKY / domain cluster zinc of each cereal, position and ID as obtained from InterProScan

Figure 3 highlights that the percentage similarity between cereal sequences is generally low. Transcription factor *WRKY*6 isoforms X1, X2 and X3 (*G. max*) show 100 % similarity, as do TF *WRKY*44, *WRKY*55 (*G. max*), WRKY factor 70-like (*Z. mays*), *WRKY1* and *WRKY62* (*S. bicolor*). Transcription factor *WRKY*30 (*G. max*) isoforms X1 and X3 share 91 % similarity, while *O. sativa japonica* Group shows 98 % similarity between isoforms X3 and X4. Transcription factor *WRKY*6 isoform X2 (*G. max*) shares 39 % similarity with *WRKY*6 isoform X2 (*S. bicolor*) and *WRKY*6 isoform X1 (*S. bicolor*). *WRKY*6 isoform X1 (*G. max*) shows 38 % similarity with *WRKY* 6 isoform X2 (*S. bicolor*) and *WRKY* 6 isoform X1 (*S. bicolor*).

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y
A																									
B	19																								
C	19	100																							
D	19	14	14																						
E	15	11	11	16																					
F	16	10	10	19	91																				
G	16	10	10	19	91	100																			
H	17	21	21	16	14	15	15																		
I	17	21	21	16	14	15	15	100																	
J	17	21	21	16	14	15	15	100	100																
K	13	15	15	20	22	25	25	18	18	18															
L	13	16	15	20	22	25	25	18	18	18	100														
M	15	14	14	22	23	28	28	16	16	16	34	34													
N	14	13	13	20	21	25	25	15	15	15	36	36	98												
O	19	16	16	25	15	18	18	16	16	16	18	18	17	16											
P	20	16	16	18	14	16	16	15	15	15	18	18	20	20	18										
Q	17	13	13	17	14	15	15	14	14	14	18	18	16	18	16	18									
R	17	13	13	17	14	15	15	14	14	14	18	18	16	18	17	18	100								
S	14	16	15	19	20	26	26	13	13	13	39	38	34	39	16	19	16	16							
T	15	16	15	19	20	26	26	13	13	13	39	38	34	38	16	19	15	15	100						
U	15	15	15	18	30	31	31	16	16	16	23	23	26	27	24	18	16	16	27	27					
V	14	15	15	18	30	30	30	16	16	16	23	23	26	27	24	18	16	16	27	27	100				
W	21	18	18	28	17	21	21	16	17	17	21	21	20	18	35	19	19	19	19	18	19	19			
X	21	18	18	28	17	21	21	17	17	17	21	21	20	18	35	19	19	19	19	18	19	19	100		
Y	18	14	14	16	15	19	19	20	20	20	20	20	18	17	14	19	36	38	17	17	23	23	15	15	
Z	17	14	14	16	14	18	18	20	20	20	20	20	19	17	14	20	36	37	17	17	23	23	15	15	100

- Legend: A:*WRKY* DNA binding domain-containing protein isoform X1 [*Zea mays*] B:*WRKY* DNA-binding transcription factor 70-like isoform X1 [*Zea mays*] C:*WRKY* DNA-binding transcription factor 70-like isoform X2 [*Zea mays*] D:*WRKY* transcription factor *WRKY51* isoform X2 [*Zea mays*] E:*WRKY* transcription factor 30 isoform X3 [*Glycine max*] F:*WRKY* transcription factor 30 isoform X1 [*Glycine max*] G:*WRKY* transcription factor 30 isoform X2 [*Glycine max*] H:*WRKY transcription* factor 55 isoform X1 [*Glycine max*] I:*WRKY* transcription factor 55 isoform X2 [*Glycine max*] J:*WRKY* transcription factor 55 isoform X3 [*Glycine max*]K:*WRKY* transcription factor 6 isoform X2 [*Glycine max*] L:*WRKY* transcription factor 6 isoform X1 [*Glycine max*] M:*WRKY* transcription factor 6 isoform X3 [*Oryza sativa japonica* Group] N:*WRKY* transcription factor 6 isoform X4 [*Oryza sativa japonica* Group] O:protein *WRKY1* isoform X2 [*Oryza sativa japonica* Group] P:*WRKY* transcription factor 23 isoform X2 [*Oryza sativa japonica* Group] Q:*WRKY* transcription factor SUSIBA2-like isoform X1 [*Sorghum bicolor*] R:*WRKY* transcription factor SUSIBA2-like isoform X2 [*Sorghum bicolor*] S:*WRKY* transcription factor 6 isoform X2 [*Sorghum bicolor*] T:*WRKY* transcription factor6 isoform X1 [*Sorghum bicolor*] U:*WRKY* transcription factor *WRKY62* isoform X2 [*Sorghum bicolor*] V:*WRKY* transcription factor *WRKY62* isoform X1 [*Sorghum bicolor*] W:protein *WRKY1* isoform X1 [*Sorghum bicolor*] X:protein *WRKY1* isoform X2 [*Sorghum bicolor*] Y:*WRKY* transcription factor 44 isoform X2 [*Glycine max*] Z:*WRKY* transcription factor 44 isoform X1 [*Glycine max*]

Fig. 3. Similarity percentage between each cereal

Motif analysis using MEME Suite identified 10 motifs across 26 *WRKY* gene sequences related to their roles in stress control. Each motif varies in location within the gene sequences and is represented by different colors (Figure 4).

Figure 4 reveals that *WRKY* DNA-binding domain-containing protein isoform X1 (*Z. mays*), WRKY transcription factor *WRKY51* isoform X2 (*Z. mays*) and *WRKY1* protein isoform X2 (*O. sativa japonica* Group) share motifs PEDGYQWRKYGQKVIKGNPYPRAYYRCTM and TGCPVRKQVZRCADDPSMLITTYEGEHNH, which are also prevalent among cereal sequences. These motifs appear adjacent in all cereal sequences. For example, *WRKY* DNA-binding 70-like isoforms X1 and X2 (*Z. mays*) share three identical motifs: LPPAALAMASTTSAAAAMLLSGSTESSDG, PEDGYQWRKYGQKVIKGNPYPRAYYRCTM and EGQQIEAQAFEASCRKPRVSVRARSESEJ. *WRKY*30 isoforms X1, X2 and X3 (*G. max*) share three identical motifs: ATELDLNSDRRAMVLAGALZZELRRLSEENRRLRGMLDQITEAYSALQEQ, KYGQKKVKGSENPRSYYKCTHPNCSVKKYKERSSDGKISEFVYKGEHNHN and EGQQIEAQAFEASCRKPRVSVRARSESEJ, although isoforms X1 and X2 have additional motifs. *WRKY* 55 isoforms X1, X2 and X3 (*G. max*) share six identical motifs: ATELDLNSDRRAMVLAGALZZELRRLSEENRRLRGMLDQITEAYSALQEQ,TQLQPQMDATEMQEVLRSSHALT MDSLYQMHQFSSARSTLQIGSMGGSDG,PEDGYQWRKYGQKVIKGNPYPRAYYRCTM,TGCPVRKQVZRCADD PSMLITTYEGEHNH, PITSSNSTTSASALSPTITLDLTQGGGPG and

ASRFGGDYPVVDMADAMFNSRSSSNNSMEFJFSPEDKSDPK. *WRKY*6 isoforms X1 and X2 (*G. max*) share seven identical motifs, and *WRKY* SUSIBA2-like isoforms X1 and X2 (*S. bicolor*) share six identical motifs, although their positions differ.

Motif similarities include PEDGYQWRKYGQKVIKGNPYPRAYYRCTM present in all cereal sequences, TGCPVRKQVZRCADDPSMLITTYEGEHNH in 23 sequences, and ASRFGGDYPVVDMADAMFNSRSSSNNSMEFJFSPEDKSDPK in only five sequences. The motifs TQLQPQMDATEMQEVLRSSHALTMDSLYQMHQFSSARSTLQIGSMGGSDG and LVDEVAAAJTNDPNFTTALAAAISSIIGE appear in nine sequences each (Figure 5).

Fig. 4. Position of each motif of each cereal

Motif	Number of motifs present in each cereal sequence
PEDGYQWRKYGQKVIKGNPYPRAYYRCTM	26
TGCPVRKQVZRCADDPSMLITTYEGEHNH	23
PITSSNSTTSASALSPTITLDLTQGGGPG	15
ATELDLNSDRRAMVLAGALZZELRRLSEENRRLRGMLDQITEAYSALQEQ	14
EGQQIEAQAFEASCRKPRVSVRARSESEJ	12
LPPAALAMASTTSAAAAMLLSGSTESSDG	10
TQLQPQMDATEMQEVLRSSHALTMDSLYQMHQFSSARSTLQIGSMGGSDG	9
LVDEVAAAJTNDPNFTTALAAAISSIIGE	9
KYGQKKVKGSENPRSYYKCTHPNCSVKKYKERSSDGKISEFVYKGEHNHN	7
ASRFGGDYPVVDMADAMFNSRSSSNNSMEFJFSPEDKSDPK	5

Fig. 5. Number of motifs present in each cereal sequence

3.4 Phylogenetic Analysis

The phylogenetic tree constructed using the neighbor-joining method with 1000 bootstrap replicates in MEGA 11 reveals two main clades marked in red and blue, representing different lineages of *WRKY* transcription factors (Figure 5). The blue clade predominantly consists of sequences from *Z. mays*, along with several from *Glycine max* and *S. bicolor* that share the same clade. Within this clade, *WRKY*1 isoform X1 from *S. bicolor* and *WRKY*1 isoform X2 from *S. bicolor* cluster closely with a bootstrap value of 100. This clade, including *WRKY*1 X2 from *O. sativa japonica*, shows a high bootstrap value of 97, indicating a strong evolutionary relationship, although not reaching 100. In comparison, these three species show a bootstrap value of 96 when compared with the *WRKY* DNA-binding domain X1 isoform from *Zea mays*.

Within the same blue clade, transcription factor *WRKY*55 isoform X1 and X3 from *Glycine max* show the lowest bootstrap values within the species group, at 86. However, both are closely related to *WRKY* isoform X2 from *Glycine max*, which shows a strong bootstrap value of 100. Additionally, *WRKY* DNA-Binding domain factor 70-like isoform X1 and X2 from *Zea mays* form a group with a high bootstrap value of 100, indicating a close evolutionary relationship within this subgroup. Conversely, in all these clusters, all species show a bootstrap value of 38 with transcription factor *WRKY WRKY*51 isoform X2 from *Zea mays*, indicating a weaker relationship among the involved species.

In the red clade, several groups show strong bootstrap support. For example, the node connecting XP_006600417.1 (*Glycine max*) and XP_003550582.1 (*Glycine max*) shows a bootstrap value of 100, while XP_021313642.1 (*Sorghum bicolor*) and XP_021310094.1 (*Sorghum bicolor*) also display a bootstrap value of 100. However, the bootstrap value for the relationship between *WRKY*30 isoform X1 (*Glycine max*) and *WRKY*30 isoform X1 (*Glycine max*) is relatively low at 73. All species within this red clade show only 37 % similarity with transcription factor *WRKY*23 isoform X2 from *Oryza sativa japonica*.

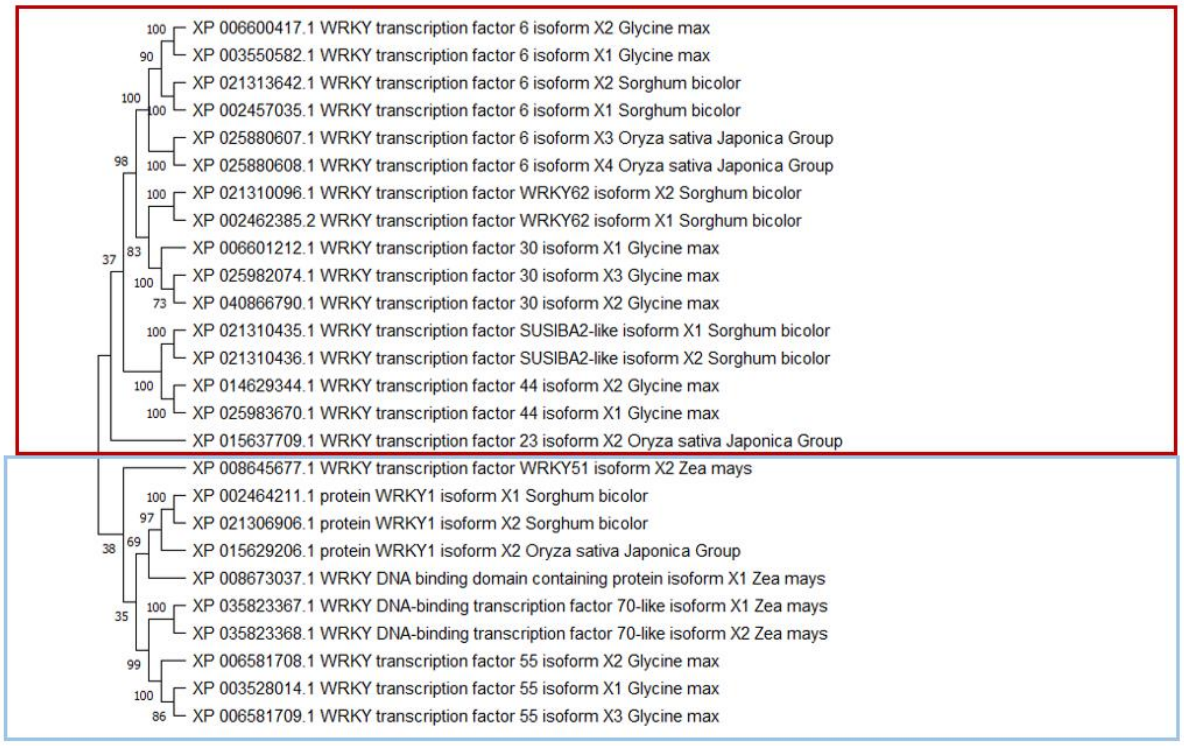

Fig. 6. Results of phylogenetic tree analysis

4. Discussion

4.1 Analysis of WRKY Gene Sequence Alignment

This study conducted a computational survey of WRKY transcription factors in four cereal crops: rice (Oryza sativa subsp. japonica), sorghum (Sorghum bicolor), soybean (Glycine max) and maize (Zea mays). Four different computational analyses were performed on 26 WRKY genes. Multiple sequence alignment revealed the presence of the conserved WRKYGQK domain in all WRKY proteins studied (Figure 1). This domain has been identified in previous studies on WRKY transcription factors [35,36]. The multiple sequence alignment also uncovered significant differences among the analyzed WRKY protein sequences. Most similar studies have also reported significant differences among the WRKY proteins investigated [37,38].

4.2 Analysis of WRKY Domains and Motifs in Cereals

WRKY genes play a crucial role in regulating various processes in plants [39,40]. Numerous studies have provided valuable information about the *WRKY* gene family in various species such as rice, tomato, cotton, Arabidopsis, pineapple and strawberry [39-43]. To our knowledge, few studies have analyzed the structure and function of *WRKY* genes across different cereal crops. In this study, five cereal sequences were classified into Group I, 15 into Group II and four into Group III. The WRKY6 transcription factor and WRKY1 protein from *Sorghum* were classified into different groups within sorghum (Table 1). According to Baillo *et al.,* (2020) [44], structural features and sequence variations align them with different WRKY groups. Transcription factor WRKY6 from *Sorghum* is placed in Group IIb because it has a single WRKY domain and a C2H2 zinc finger motif typical of Group IIb members, which are generally involved in responses to abiotic and biotic stresses. In contrast, WRKY1 from sorghum is classified into Group IId despite also having a single WRKY domain; as it possesses specific sequence variations and conserved motifs characteristic of Group IId members, which are often associated with plant development regulation and stress responses. These structural differences drives their classification into distinct groups and assignment of function (Figure 2).

Transcription factors play a significant role in responding to abiotic and biotic stress by binding to DNA sequences to activate or suppress the expression of target genes. Abiotic stresses include extreme temperatures (both high and low), salinity, drought and oxidative stress, which damage biochemical and physiological processes in plants. Previous studies have shown that WRKY transcription factors are involved in responding to abiotic stress [45]. Many *WRKY* genes are upregulated under cold, drought, high salinity, heavy metal and heat stress conditions to enhance tolerance to abiotic stress in plants.

We believe that the motifs as structural or functional elements, act as binding sites for other molecules. In the context of WRKY transcription factors, these motifs are specific amino acid sequences crucial for the proteins function in regulating gene expression in response to various stimuli [45]. The conservation of motifs across species and isoforms highlights their importance in maintaining WRKY transcription factor function. Motif analysis identified *PEDGYQWRKYGQKVIKGNPYPRAYYRCTM* as a dominant motif frequently found in all cereal sequences, often adjacent to TGCPVRKQVZRCADDPSMLITTYEGEHNH, indicating high conservation of these sequences in cereals. This motif is known to interact with W-box elements in the promoters of genes involved in defense responses to pathogens [46]. Thus, this motif plays a crucial role in modifying plant responses to pathogen attacks and abiotic stresses such as drought, high salinity and extreme temperatures [47] (Figure 4 and 5).

4.3 Phylogenetic Analysis

The phylogenetic tree was constructed based on species, WRKY transcription factor similarities and motif content (Figure 6). According to this phylogenetic tree, sequences are grouped without regard to isoform forms, with no clades or grouping based on isoenzymes observed. However, species with the same transcription factors show very close relationships both interspecifically and intraspecifically. This can be seen in WRKY transcription factors from *Glycine max*, *Sorghum bicolor* and *Oryza sativa japonica* Group. According to Yousfi *et al.,* (2005) [48], phylogenetic analysis of *WRKY* across various plant species indicates that these factors often cluster based on function and similar motif sequences rather than isoenzymes. Additionally, cereal sequences with similar motifs cluster together; for example, all transcription factor 6 sequences from *Glycine max*, *Sorghum bicolor* and O*ryza sativa japonica* Group have nearly identical motifs. However, species from the same genus are not necessarily grouped together. For instance, the O*ryza sativa japonica* factor 6 sequences are not grouped with *WRKY* factor 23 due to lower motif similarity. The transcription factor 6 sequences from O*ryza* sativa shows a close relationship with those from *Glycine max* and *Sorghum bicolor*, indicating that cereal sequences are also grouped according to their transcription factors (Figures 3 and 6). This research supports the idea that the evolutionary relationships between transcription factors are influenced by their genus, species, functions and motifs rather than their isozyme classification.

5. Conclusions

This study successfully characterized 26 *WRKY* gene sequences from cereal species such as *Oryza sativa japonica* Group, *Glycine max*, *Sorghum bicolor* and *Zea mays*. Sequence alignment using ClustalW revealed the conserved region *WRKY*GQK in *WRKY* genes, which is crucial for understanding the conserved function of these genes in cereal species. The alignment results confirm the presence of the WRKYGQK domain in all analyzed WRKY proteins, highlighting the necessity of this domain for *WRKY* gene biological activity. Phylogenetic analysis shows that *WRKY* genes are more likely clustered based on plant species rather than isoenzymes, indicating that plant species play a major role in the evolution of these genes. The phylogenetic tree clusters sequences based on *WRKY* transcription factor similarities and motif content, supporting the theory that these genes cluster based on function and similar motif sequences, rather than isoenzymes.

The motif WRKYGQK was found in all *WRKY* genes, with the motif PEDGYQWRKYGQKVIKGNPYPRAYYRCTM being dominant across all cereal sequences. These findings suggest that conserved motifs like WRKYGQK are active sites necessary for *WRKY* transcription factors to bind to target gene promoters and regulate their expression in response to abiotic and biotic stress. Additionally, the motif PEDGYQWRKYGQKVIKGNPYPRAYYRCTM, is frequently found adjacent to TGCPVRKQVZRCADDPSMLITTYEGEHNH, indicating a high level of conservation of these sequences in cereals.

Classification of *WRKY* genes into Groups I, II and III based on the number of domains and zinc finger presence reveals different functional roles for WRKY transcription factors in stress response pathways. In this study, five cereal sequences were classified into Group I, fifteen into Group II and four into Group III, while two sequences were not classified into any group. WRKY transcription factor 6 from *Sorghum bicolor* was placed into different groups, namely Group IIb and IId, due to its distinct zinc finger structure compared to the same species.

Overall, *WRKY* genes are hypothesized to be potential candidates for enhancing stress tolerance and plant resilience through biotechnological approaches such as transgenic technology or breeding.

Further research focusing on the regulatory mechanisms of *WRKY* genes is important for agricultural applications, including genetic manipulation to improve stress resistance and exploring interactions between WRKY motifs and target gene promoters at the molecular level. Future research may also focus on species-specific variations in *WRKY* genes to gain a deeper understanding of the evolution and function of these transcription factors in various environmental contexts.

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