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The Role of *OsWRKY* Genes in Rice When Faced with Single and Multiple Abiotic Stresses

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Abstract: The WRKY genes are one of the largest families of transcription factors (TFs) and play a crucial role in certain processes in plants including stress signaling, regulation of transcriptional reprogramming associated with stress responses, and other regulatory networks. This study aims to investigate the WRKY gene family in the C_3 model plant, *Oryza sativa* L., using a genome-wide in silico expression analysis. Firstly, 104 WRKY TF family members were identified, and then their molecular properties and expression signatures were analyzed systematically. In silico spatio-temporal and hormonal expression profiling revealed the roles of *OsWRKY* genes and their dynamism in diverse developmental tissues and hormones, respectively. Comparative mapping between *OsWRKY* genes and their synteny with C_4 panicoid genomes showed the evolutionary insights of the WRKY TF family. Interactions of *OsWRKY* coding gene sequences represented the complexity of abiotic stress (AbS) and their molecular cross-talks. The expression signature of 26 novel candidate genes in response to stresses exhibited the putative involvement of individual and combined AbS (CAbS) responses. These novel findings unravel the in-depth insights into *OsWRKY* TF genes and delineate the plant developmental metabolisms and their functional regulations in individual and CAbS conditions.

Keywords: abiotic stress; comparative mapping; GWAS; *Oryza sativa*; *OsWRKY*; transcription factor

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1. Introduction

As sessile organisms, plants are continuously exposed to adverse environmental conditions which may cause deleterious impacts on their growth, development, and productivity. Abiotic stresses (AbS) are predominant among various environmental stresses, which include drought, low temperature or cold stress, salinity, submergence, heavy metal, and other forms of oxidative stress such as radiation. At present, global agriculture is facing a serious threat from climatic changes, which is another aggravating challenge that affects the sustainability and productivity of crop plants [1]. Plants have well-developed defense responses to ensure survival under these environmental stresses and exhibit stress avoidance/stress tolerance through acclimation and adaptation mechanisms [2]. On deeper insight of stress, initiation of complex abscisic acid (ABA) -dependent and/or -independent signal transduction pathways and its manifestation at physiological, molecular, and metabolic responses that ultimately elevate the stress tolerance in plants [3].

Transcription factors (TFs) are the pivotal players involved in the stress signaling pathways, transcriptional reprogramming, cell division, plant growth, development, and stimulating the abiotic stress-responsive (AbSR) genes [4,5]. The TFs may afford signaling cascades or gene networks, in which they regulate other TFs and/or functional regulatory elements through their specific binding sequences [6,7]. The plant genome devotes approximately 7% putative TFs [8] that are classified into 58 TF families [9]. Among these, WRKY is the seventh-largest family of TFs (https://grassius.org/, accessed on 4 December 2020) that are mainly found in higher plants with 74 members in *Arabidopsis* and up to 104 members predicted in *Oryza sativa* [10]. WRKY TF domain is composed of a conserved WRKYGQK sequence motif and a CX5-8CX25-28HX1-2C or CX4-5CX22-23HXH (metal-chelating zinc finger) motif [11]. WRKY TFs contain 60 highly conserved amino acids with a WRKY motif sequence at its amino-terminal end and a putative zinc finger domain at its carboxy-terminal end [12]. The WRKY family could specifically bind on the W-box promoter region with consensus sequence (C/T) TGAC [T/C], which targets the downstream genes and stimulates their expression dynamism. In addition, WRKY TFs can bind to both W-box and a sugar-responsive (SURE) *cis*-element (TAAAGATTACTAATAGGAA) and activate the transcription machinery of downstream genes [13].

To the best of our knowledge, WRKY TFs are involved in functional regulation of diverse physiological and molecular processes including pollen development and its function [14], seed dormancy [15], trichome development [16], seed development [13,16,17], flowering time and plant height [18], somatic embryogenesis [19,20], biomass [21,22], secondary metabolite biosynthesis [13,23–25], hormone signaling [26] and leaf senescence [27–29]. Most importantly, WRKY TFs have been shown to get activated in response to different biotic [10,30] and AbS [31,32], including pathogen infection [33,34], oxidative stress [35], drought, cold, high salinity [36], wounding [37], freezing [38], bacterial infection [39–41], viral attack [39,42], fungal invasion [39,43,44], defense against oomycetes [45,46], carbohydrate anabolism, and secondary metabolism [13]. Furthermore, AbS induces the activity of various WRKY proteins, which function in synchronization to confer resistance against certain stress or provide a combinatorial effect on combined stress resistance. Some of the WRKY TFs can be differentially expressed, regulating the expression of related genes and promoting signal transduction machinery. In wheat, out of the 15 WRKY genes, 8 genes were transcribed in response to NaCl, heat, polyethylene glycol (PEG), and cold [47]. In rice, the majority of the WRKY genes show variable responses towards PEG, salinity, cold, and heat stresses [48]. Elevated expression of 18 WRKY genes in the roots of NaCl treated Arabidopsis plants were confirmed via microarray profiling [49]. The expression profiling and functional identifications of WRKY TFs in most studies are generally based on genetic transformation, real-time fluorescence quantitative PCR, and transcriptome analyses. Hence, WRKY genes can function effectively in different abiotic stress responses or tolerances in rice and various crop plants.

The potential role of WRKY TF family members in various molecular, physiological and biological processes has been studied extensively in a variety of crop plants [50–54]. However, a systematic view of WRKY TFs in the C3 model plant, *O. sativa* (*OsWRKY*) is still inadequate. In view of its importance, high-throughput in silico approaches have been used to identify the potential AbSR—*OsWRKY* TF family members for the first time. This study provides the functional aspects of these AbSR—*OsWRKY* TFs and spotlights potential candidates for further characterization toward representing their functional role in AbS dynamism.

2. Materials and Methods

2.1. In Silico Mining and Meta-Analysis of WRKY TF Genes in O. sativa

The WRKY family members and their encoding gene sequences of *O. sativa* were retrieved from the GRASSIUS Grass Regulatory Information Server (http://grassius.org/grasstfdb.html, accessed on 4 December 2020) [55]. *OsWRKY* TF family members and their RAP ID/locus ID were collected and used for further analyses. Furthermore, *WRKY* genes

with their IDs were used to fetch the corresponding genomic, transcriptomic, and coding sequences along with their chromosomal positions were collected from the RGAP (Rice Genome Annotation Project Database) (http://rice.plantbiology.msu.edu/, accessed on 24 December 2020) [56].

2.2. Spatio-Temporal and Phytohormone Expression Analysis

OsWRKY genes were exported to the spatio-temporal (RXP_0001) dataset and plant hormone (RXP_1001~RXP_1012) dataset of rice expression profile database (RiceXPro) (http://ricexpro.dna.affrc.go.jp/, accessed on 16 January 2020) [57] for analyzing the spatio-temporal gene expression profile covering different tissues/organs and cell types at various developmental stages, and plant hormone responses, respectively using publicly available microarray.

2.3. Gene Features and Phylogenetic Analysis

Gene properties including amino acid length, molecular weight (M.Wt), isoelectric point (pI), instability index, aliphatic index, and grand average hydropathicity (GRAVY) were predicted using the online ExPASy proteomics server (http://web.expasy.org/protparam/, accessed on 2 February 2020) [58]. The *OsWRKY* TF family members and their respective amino acid sequences in other C_4 panicoid sequenced grass species such as foxtail millet (*Setaria italica*), sorghum (*Sorghum bicolor*), and maize (*Zea mays*) were also identified by BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Genes, accessed on 8 February 2020) analysis. The accession numbers of newly predicted WRKY TFs family members from C_4 grass species were assigned as *SiWRKY* (*S. italica*), *SbWRKY* (*S. bicolor*), *ZmWRKY* (*Z. mays*) TF family members and their identity score values were tabulated (Supplementary Table S1). The predicted WRKY TF gene sequences confirmed the presence of WRKY DNA—binding domain and hAT family C-terminal dimerization domain by HMMSCAN (Supplementary Table S2). The amino acid sequences of *OsWRKY* along with *SiWRKY, SbWRKY, ZmWRKY* were imported into MEGA v7.0 (Philadelphia, PA, USA) [59] and multiple sequence alignment was performed using ClustalW. The parameters used in the alignment were as follows: gap opening: 10.00, and gap extension: 0.10. The alignment file was imported to construct a phylogenetic tree by the maximum-likelihood method and bootstrap analysis was performed with 1000 replicates.

2.4. Gene Structure Analysis and Gene Ontology Annotation

Understanding the gene organization will aid to reveal the function, regulation, and evolution of genes. Arrangements of exons and introns were predicted by comparing the coding sequences with their genomic sequences using Gene Structure Display Server (GSDS) v2.0, a web-based bioinformatics tool (http://gsds.cbi.pku.edu.cn/, accessed on 16 February 2021) [60]. Potential candidate genes and their corresponding RAP IDs were subjected to the ShinyGO v0.61 database (http://bioinformatics.sdstate.edu/go/, accessed on 7 March 2021) to obtain gene ontology (GO) annotation against *O. sativa* subsp. *japonica*. GO enrichment was calculated by the *p*-value cut-off (FDR) at 0.01 for the genes.

2.5. Molecular Interactome and Enrichment Analysis

Protein-Protein Interaction (PPI) analysis was performed using STRING v11.0 (https://string-db.org/, accessed on 20 March 2021) [61] with a high confidence score of 0.7. The functional enrichment analysis of the interactome was done through the level of 0.01. Active interaction based on the various sources, including text mining, experiments, gene fusion, databases, and co-expression, and an interaction score > 0.4 were applied to construct the PPI network. This interactome map was used to identify the physical and functional role of the key candidate genes involved.

2.6. Gene Synteny Analysis

In order to unravel the genomic distribution of WRKY TFs, comparative mapping/gene synteny analysis was performed. *OsWRKY* and their orthologous genes in C4 grasses such as *S. italica*, *S. bicolor,* and *Z. mays* were identified by Gramene-BLASTP (reciprocal) analysis of the gene sequences against these panicoid genomes. All orthologous gene sequences and all hits with E-value 1e−0 (1×10^0) and minimum 60% similarity were treated as significant. The chromosomal synteny between *O. sativa* and these C4 grass species was then visualized by Circos v0.55 (Switzerland) [62].

3. Results

3.1. Identification of the WRKY Family Transcription Factors in Rice Genome

The GRASSIUS Grass Regulatory Information Server identified a total of 104 *OsWRKY* TF family members (Supplementary Table S3). These 104 genes were subjected to RiceXPro for meta-expression analysis (Supplementary Figure S1). Based on the heatmap profiling, 26 *OsWRKY* TF novel candidate genes were identified and these players are significantly involved in individual and CAbS responses (Table 1). Notably, these genes were localized in all the rice chromosomes except the 10th chromosome, which revealed these players divergence of chromosomes in the rice genome.

Table 1. *OsWRKY* TF family members and their attributes.

S. NO	Gene Name	RAP ID	Start	End	Chr. No	UniProt ID
1	*OsWRKY*1	Os01g0246700	4356383	4340849	1	Q0JQ43
2	*OsWRKY*6	Os03g0798500	22731943	22733240	3	Q94D50
3	*OsWRKY*10	Os01g0186000	26688416	26687377	1	Q0JKQ9
4	*OsWRKY*11	Os01g0626400	25009453	25012236	1	Q9FE35
5	*OsWRKY*12	Os01g0624700	29723065	29720923	1	Q5JLU2
6	*OsWRKY*14	Os01g0730700	30604295	30608077	1	Q942D2
7	*OsWRKY*23	Os01g0734000	36194611	36193840	1	Q6IEM5
8	*OsWRKY*24	Os01g0826400	42946753	42948750	1	Q5JM93
9	*OsWRKY*28	Os06g0649000	26283914	26280253	6	Q0DZ26
10	*OsWRKY*29	Os07g0111400	28726783	28730933	7	Q6Z8E9
11	*OsWRKY*32	Os02g0770500	12394669	12396898	2	Q10LT9
12	*OsWRKY*37	Os04g0597300	31326926	31323190	4	Q9AUV7
13	*OsWRKY*45	Os05g0322900	30132491	30136547	5	Q0JAI8
14	*OsWRKY*49	Os05g0565900	4999626	4998210	5	Q65WY5
15	*OsWRKY*55	Os03g0321700	23530499	23529423	3	Q6IEN3
16	*OsWRKY*66	Os02g0698800	2958991	2963006	2	Q5VMX9
17	*OsWRKY*71	Os07g0583700	23659625	23654076	7	Q84ZS7
18	*OsWRKY*73	Os07g0680400	28832398	28828793	7	Q7XHX5
19	*OsWRKY*74	Os08g0198000	5669406	5663578	8	Q0J7F5
20	*OsWRKY*79	Os08g0386200	18220041	18222408	8	Q6ZA22
21	*OsWRKY*82	Os09g0334500	10128825	10131136	9	Q6ERI5
22	*OsWRKY*83	Os09g0417600	14977713	14975932	9	Q6EPZ2
23	*OsWRKY*85	Os09g0481700	18501264	18496949	9	Q0J0V4
24	*OsWRKY*92	Os11g0117500	789030	787542	11	Q2RBB8
25	*OsWRKY*94	Os11g0490900	17352085	17355820	11	Q2R432
26	*OsWRKY*101	Os12g0116700	825793	824302	12	Q2QYJ6

3.2. Spatio-Temporal Expression Analysis of OsWRKY

To detect dynamic changes in spatio-temporal expression level of 104 *OsWRKY* TF genes in 48 different tissue/organ, specific meta-profiling was performed and observed at diverse developmental stages of rice plants under natural field conditions (Supplementary Figure S1). Among the 104 *WRKY* TF genes, 26 key players (listed in Table 1) showed higher expression pattern in diverse tissues and organs such as leaf sheath (vegetative—12:00; 00:00; reproductive—12:00; 00:00), leaf blade (vegetative—12:00; 00:00, reproductive—12:00; 00:00, ripening—12:00; 00:00), root (vegetative—12:00; 00:00, reproductive—12:00; 00:00),

inflorescence (0.6–1.0, 3.0–4.0, 5.0–10 mm), stem (ripening—12:00; 00:00), ovary (01, 03 days after flowering (DAF)), and endosperms (07, 10, 14, 28 and 42 DAF (Figure 1). *OsWRKY* showed lower expression in stem (reproductive—12:00; 00:00), lemma (1.5–2.0, 4.0–5.0, 7.0 mm floret), palea (1.5–2.0, 4.0–5.0, 7.0 mm floret), anther (0.3–0.6, 0.7–1.0, 1.2–1.5, 1.6–2.0 mm), ovary (05 07 DAF), and embryo (07, 10, 14, 28 and 42 DAF) (Figure 1) as imputed by RiceXPro, based on the available *OsWRKY* TF family members field RNA-Seq data.

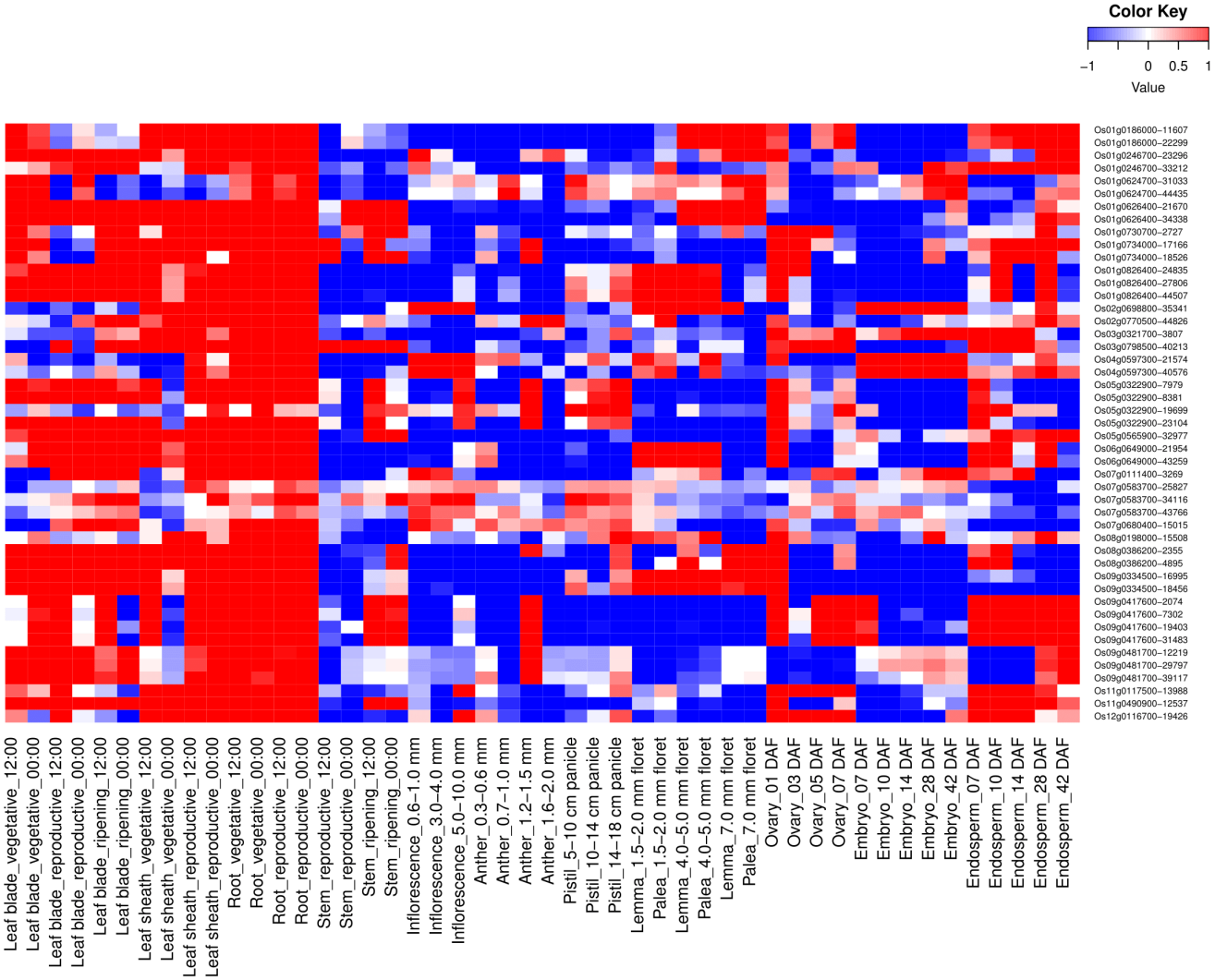

Figure 1. Heatmap of 26 potential *OsWRKY* TF family members showing the spatio-temporal gene expression profile of tissue/organ types at different developmental stages which are differentially expressed under natural field conditions. The red color indicates up-regulation, the blue color indicates down-regulation, and the white color indicates no expression. The color bar at the right side top represents the level of expression value, where −1 and 1 represent the down- and up-regulation of AbS responsible *OsWRKY* TF family members.

3.3. Phytohormone Expression Profiling

Twenty-six *OsWRKY* TF genes showed phytohormonal expression profiling in various time points such as 15 min, 30 min, 1 h, 3 h, 6 h, and 1 h, 3 h, 6 h, 12 h in root and shoot, respectively. In the shoot, auxin and jasmonic acid (JA) showed high-level expression, and a negligible level of expression was observed in abscisic acid (ABA), cytokinin (CK), gibberellins (GA), and brassinosteroid (BRs) (Figure 2) at various time points. In the root, all these 26 genes were found to show higher expression in auxin, CK, and JA and lower expression in ABA, GA, BRs hormone expression levels across all the time points (Figure 3).

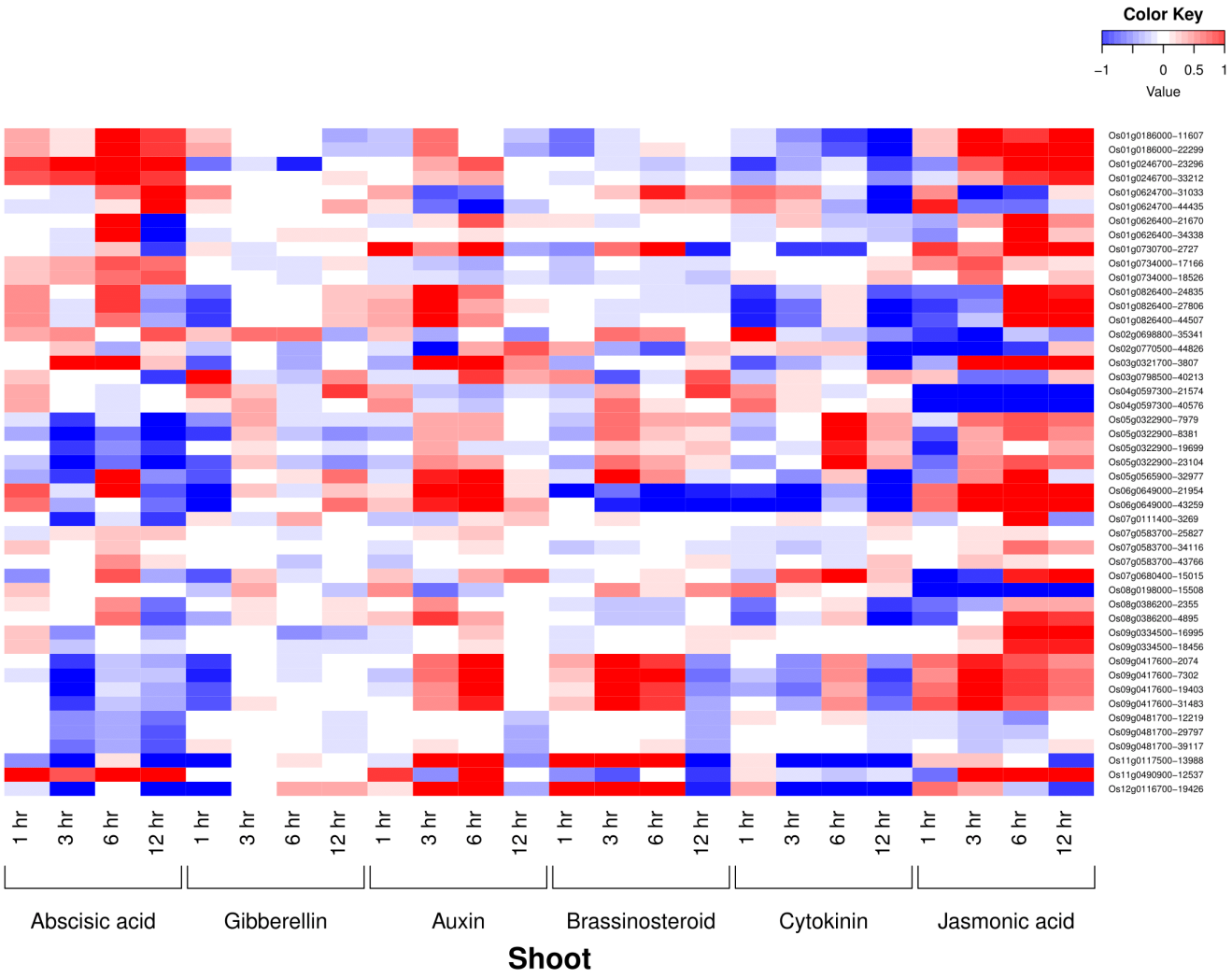

Figure 2. Heatmap representing the potential 26 AbSR—*OsWRKY* TF family members and their phytohormonal expression pattern in the shoot which are differentially regulated across the entire growth in the field conditions. The red color indicates up-regulation, the blue color indicates down-regulation, and the white color indicates no expression. The color bar at the right side top represents the level of expression value, where −1 and 1 represent the down- and up-regulation of AbS responsible *OsWRKY* TF family members.

3.4. OsWRKY TF Genes with Their Properties

The candidate genes and their properties such as amino acid length, M. Wt, pI, aliphatic index, instability index, GRAVY, and subcellular localization of the *OsWRKY* were analyzed and are given in Table 2. Among the 26 *OsWRKY* genes, *OsWRKY*28 was the smallest gene with 190 amino acids whereas *OsWRKY*74 was the largest one with 862 amino acids. The pI ranged from 9.48 (*OsWRKY*23) to 10.06 (*OsWRKY*1) and the molecular weight of the genes also varied according to gene size ranging from 20.49 kDa (*OsWRKY*28) to 97.37 kDa (*OsWRKY*74) (Table 2). The variation in the physiochemical properties of genes deciphered the presence of putative novel variants. Notably, many of the players were localized in the nucleus and it revealed that these *OsWRKY* TFs were involved in gene transcriptional and several biosynthesis processes.

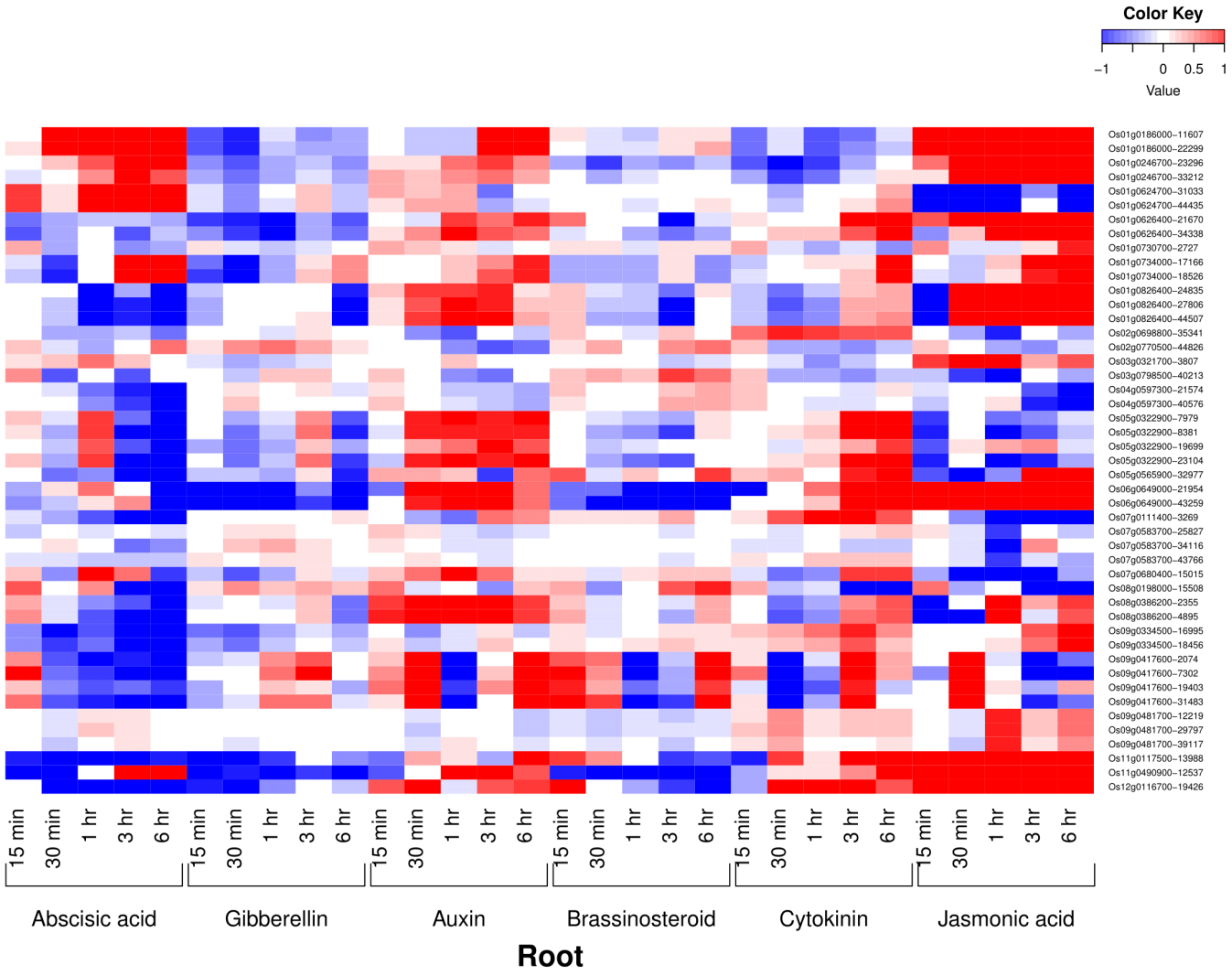

Figure 3. Heatmap showed the potential 26 AbSR—*OsWRKY* TF family members and their phytohormonal expression pattern in roots which are differentially regulated across the entire growth in the field conditions. The red color indicates up-regulation, the blue color indicates down-regulation, and the white color indicates no expression. The color bar at the right side top represents the level of expression value, where −1 and 1 represent down- and up-regulation respectively of AbS responsible *OsWRKY* TF family members.

3.5. Phylogenetic Analysis of WRKY TFs

Retrieved WRKY amino acid sequences were imported into MEGA v7.0 software (Philadelphia, PA, USA) and the unrooted phylogenetic tree was constructed by the maximum-likelihood method to study the evolutionary organization of the potential 26 WRKY TF family members (Figure 4). The unrooted tree confirmed the homology between the *OsWRKY* TF family members with *SiWRKY*, *SbWRKY*, and *ZmWRKY* using phylogenetic tree analysis.

Table 2. Details of *OsWRKY* genes and their properties.

S. No	Gene Name	RAP ID	Nt L	aa L	M. Wt	pI	AI	II	GRAVY	SL
1	*OsWRKY*1	Os01g0246700	1275	425	45,967.1	10.0694	68	40.54	−0.383	Ct
2	*OsWRKY*6	Os03g0798500	741	247	26,500.7	7.6528	68.21	55.29	−0.549	Cyto
3	*OsWRKY*10	Os01g0186000	825	275	29,056.4	7.4157	60.33	43.9	−0.4	N
4	*OsWRKY*11	Os01g0626400	1140	380	39,856	7.0634	49.45	63.38	−0.731	N
5	*OsWRKY*12	Os01g0624700	738	246	25,832.6	8.2902	54.37	43.51	−0.424	Ct
6	*OsWRKY*14	Os01g0730700	765	255	27,796.2	6.9031	66.14	47.02	−0.656	N
7	*OsWRKY*23	Os01g0734000	588	196	21,158.8	9.4863	59.18	63.57	−0.624	N
8	*OsWRKY*24	Os01g0826400	1233	411	45,109.9	4.6849	60	59.53	−0.694	N
9	*OsWRKY*28	Os06g0649000	570	190	20,495.6	8.492	64.07	50.13	−0.375	Cyto
10	*OsWRKY*29	Os07g0111400	1521	507	51,766.5	6.1521	57.67	48.26	−0.354	N
11	*OsWRKY*32	Os02g0770500	1071	357	36,,383.7	4.9404	56.83	55.42	−0.431	N
12	*OsWRKY*37	Os04g0597300	945	315	32733.4	5.869	60.83	69.16	−0.416	N
13	*OsWRKY*45	Os05g0322900	1545	515	53,042.3	5.3217	57.43	47.7	−0.415	PM
14	*OsWRKY*49	Os05g0565900	585	195	21,400.7	6.269	52.01	52.32	−0.579	Ct
15	*OsWRKY*55	Os03g0321700	996	332	34,901.2	4.3811	57.73	65.5	−0.507	N
16	*OsWRKY*66	Os02g0698800	1176	392	41,403.5	6.1909	51.33	52.47	−0.646	N
17	*OsWRKY*71	Os07g0583700	1857	619	66,163.6	6.3317	58.75	51.59	−0.683	N
18	*OsWRKY*73	Os07g0680400	1002	334	34,837.5	6.5945	54.26	70.97	−0.471	N
19	*OsWRKY*74	Os08g0198000	2586	862	97,370.3	6.4311	80.14	41.56	−0.41	N
20	*OsWRKY*79	Os08g0386200	960	320	33,550.3	6.6611	67.4	41.48	−0.316	N
21	*OsWRKY*82	Os09g0334500	1086	362	37,944.1	6.4041	64.21	53.99	−0.428	N
22	*OsWRKY*83	Os09g0417600	984	328	34,780.2	8.0535	68.69	50.66	−0.496	N
23	*OsWRKY*85	Os09g0481700	1902	634	68,280.1	6.0112	51.66	64.68	−0.712	N
24	*OsWRKY*92	Os11g0117500	963	321	35,915.9	6.417	72.25	58.59	−0.672	N
25	*OsWRKY*94	Os11g0490900	729	243	25,857.2	9.3347	46.45	55.93	−0.778	N
26	*OsWRKY*101	Os12g0116700	966	322	35,749.5	6.3705	69.31	60.38	−0.723	N

Nt L, Nucleotide length; aa L, amino acid length; M. Wt, Molecular weight; pI, Isoelectric point; AI, Aliphatic index; II, Instability index; GRAVY, Grand average of hydropathicity, SL, Subcellular localization; Cyto, Cytosol; Ct, Chloroplast; N, Nucleus; PM, Plasma membrane.

3.6. Gene Organization Analysis

Gene structure analysis revealed the number and distribution of exons and introns in the *OsWRKY* TF genes (Figure 5). The distribution of introns ranged from one to seven amid exonic sequences which may be due to evolutionary changes that have occurred in the *OsWRKY* TF family members. The majority of the genes contained two introns, whereas six genes (*OsWRKY*14, *OsWRKY*23, *OsWRKY*55, *OsWRKY*79, *OsWRKY*83, and *OsWRKY*94) had only one intron. A maximum of seven introns was found to be present in *OsWRKY1* (Figure 5).

3.7. Functional GO Analysis of OsWRKY TFs

OsWRKY TF genes and their functional ontology were predicted by the ShinyGO database which showed the involvement of these genes in various biological processes and molecular functions. *OsWRKY* novel candidate genes were imputed to be involved in stimulus, chemical, regulation of transcription, metabolic and biosynthetic processes (Figure 6). The significant molecular functions of these candidate genes were encoded for different types of sequence-specific, DNA, heterocyclic and regulatory region binding activities (Figure 7).

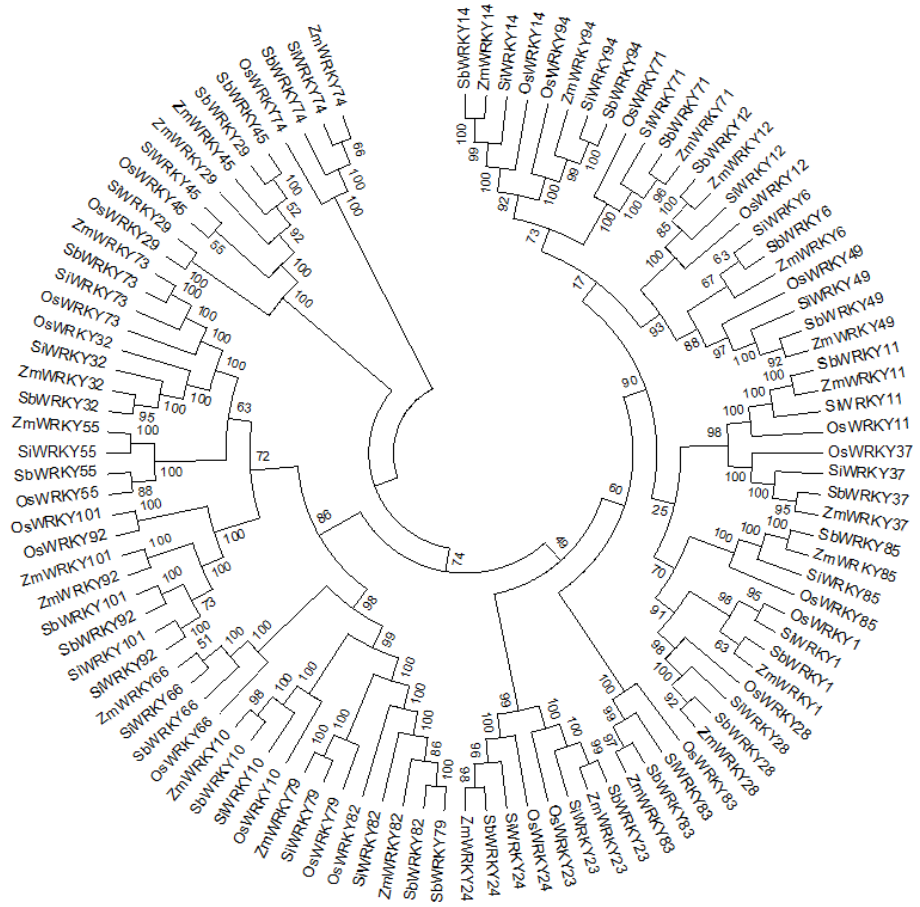

Figure 4. Phylogenetic relationships of 26 potential *OsWRKY* genes of *O. sativa*, *S. italica*, *S. bicolor*, and *Z. mays*. The amino acid sequences were aligned by ClustalW at the MEGA *v*7.0 program with the unrooted maximum likelihood method. The evolutionary tree was constructed with bootstrap confidence values from 1000 iterations.

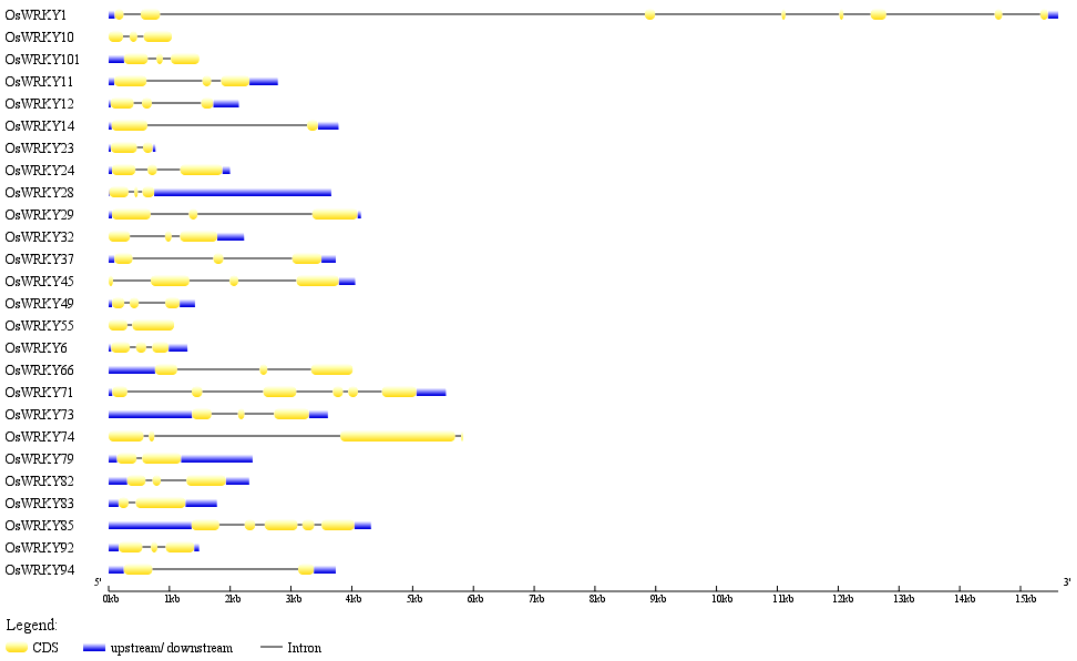

Figure 5. Structural organization of *OsWRKY* TF genes. Introns, exons, and untranslated regions (UTR) are represented by black lines, yellow, and blue rectangles. The scale bar denotes the size of the gene.

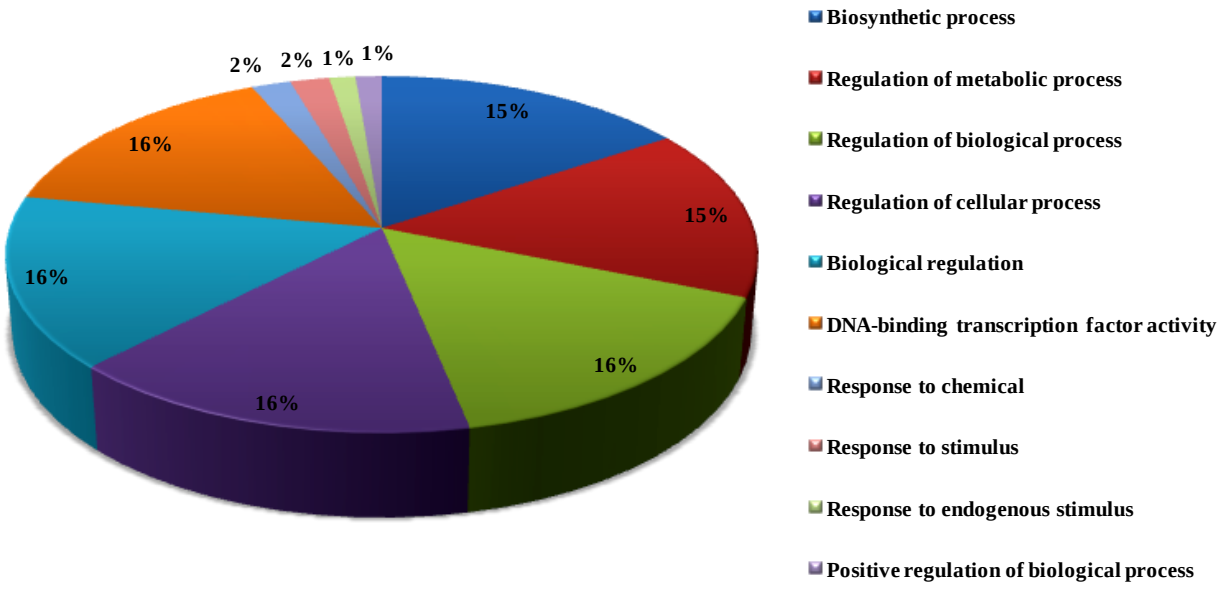

Figure 6. ***OsWRKY*** **genes and their biological functions**. The biological function was imputed according to the significance level (*p*-value FDR cutoff).

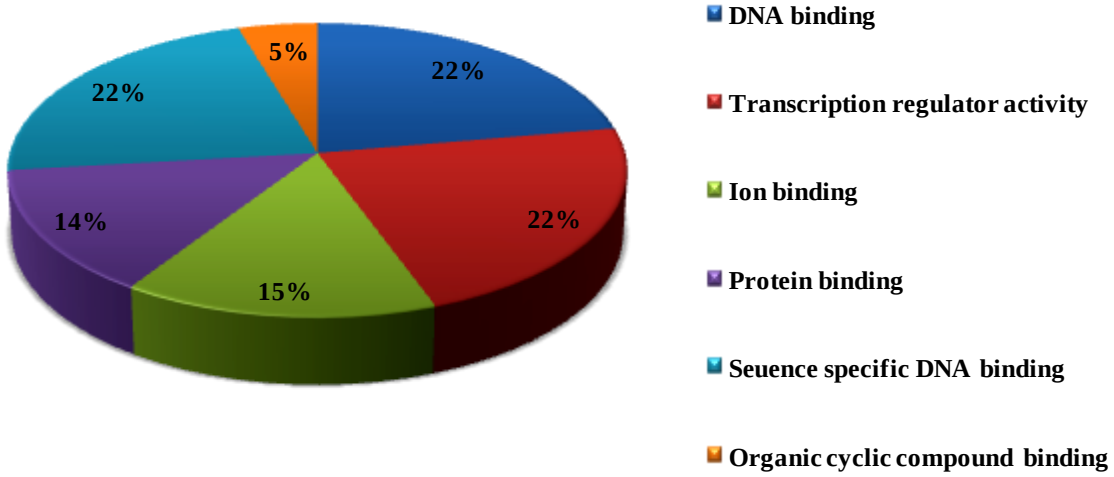

Figure 7. ***OsWRKY*** **genes and their molecular function**. The molecular function was imputed according to the significance level (*p*-value FDR cut-off).

3.8. OsWRKY Gene Interaction Network Analysis

Potential 26 WRKY TF family encoding genes were obtained from *O. sativa* ssp. *Japonica* AbSR *OSWRKY* TF genes and molecular interaction network was analyzed using the STRING *v*11.0 database. The gene network had 46 nodes, 74 weighted edges, and an enrichment *p*-value score <0.01 (Figure 8). The average nodal degree between the neighboring genes was 3.22. This interaction network revealed the complexity of AbSR *OsWRKY*, hence it proved the nature of multi-gene.

3.9. Orthologous Relationships of OsWRKY Genes

Gramene—BLASTP analysis revealed chromosomal collinearity among 26 potential *OsWRKY* genes with those of C4 panicoid grass species such as *S. italica*, *S. bicolour*, and *Z. mays*. The chromosomal ideogram exhibited the maximum relationship that occurred between *O. sativa* and C4 grass plant species [26 *OsWRKY* (100%)] (Figure 9A–C; Supplementary Tables S4–S6).

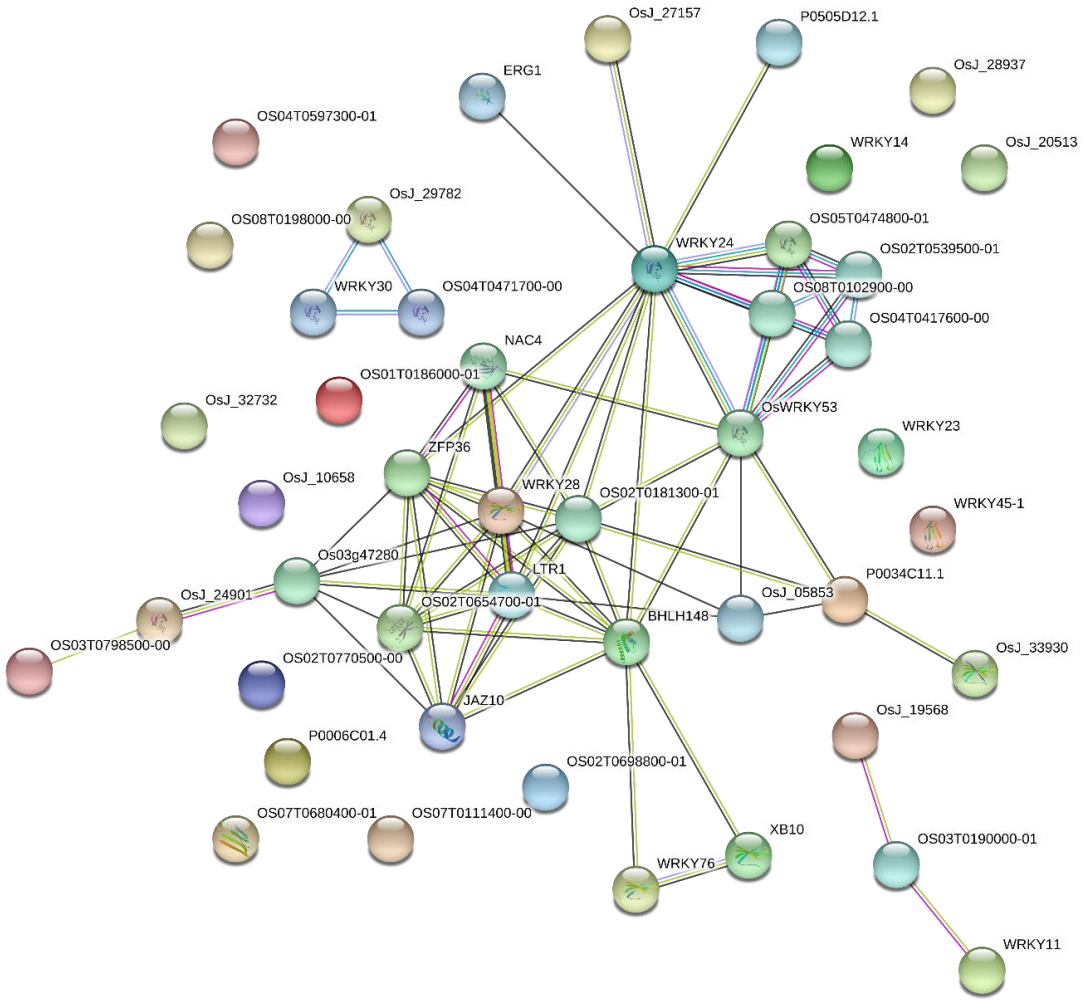

Figure 8. Molecular interaction network of 26 *OsWRKY* genes involved in various stress responses. The line thickness denotes the level of interaction between genes and colored lines between the genes denote various types of the interaction network. Black color, gene co-occurrence; red color, gene fusion; pink color, experimentally determined; violet color, gene homology; green color, gene neighborhood; blue color, gene co-occurrence. Gene nodes containing ribbon structures represent the presence of gene 3D structural information.

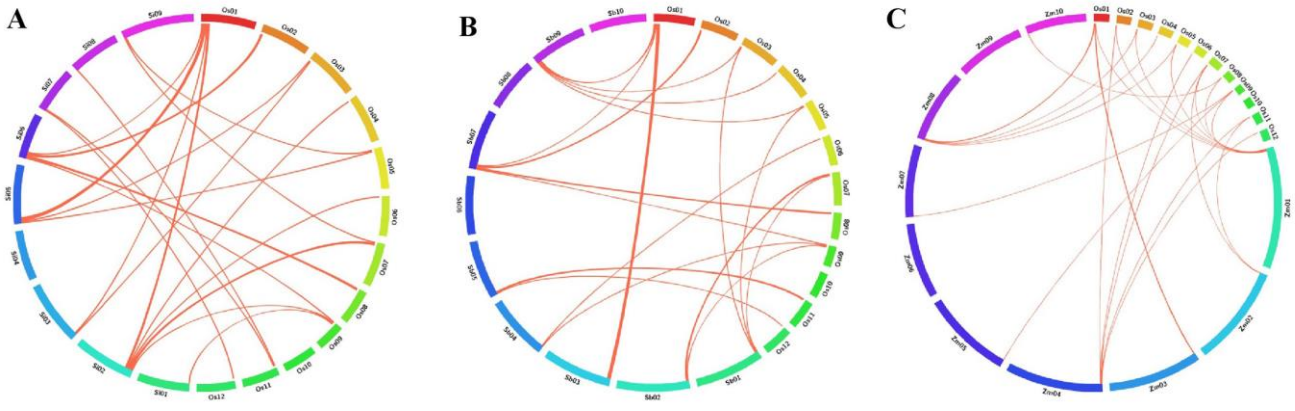

Figure 9. Comparative orthologous relationship of WRKY genes among (A) rice and foxtail millet, (B) rice and sorghum, (C) rice and maize. All segments denote chromosome and red links represent the genomic regions with a corresponding target chromosome.

4. Discussion

WRKY TFs are a class of DNA-binding proteins that play a major role in physiological processes, plant growth and development, signal transduction, senescence, seed dormancy, responses to a diverse biotic and AbS, biosynthesis and hormonal regulations and stress signaling through auto- and cross-regulation [63–65]. Compared to biotic stresses, so far, only limited information is available on the WRKY TF family members' role in AbS. Considering the importance of *WRKY* TF genes from various plant species and their crucial roles under various environmental conditions, it remains a big challenge to unveil their mechanisms in AbS. The functions of *WRKY* TF genes in defense signal transduction pathways came from the analysis of dicot plants, such as tomato, Arabidopsis, potato, and tobacco, and to date, less information was reported in rice and other monocot plants. Few studies have demonstrated that many *WRKY* genes are predominantly expressed in response to AbS such as cold, salinity, drought, flooding and submergence [66,67], extreme levels of light (high and low), sugar starvation [63], phosphate deprivation [68], radiation (UV-B and UV-A) [69], and wounding [70]. However, the mode of action of WRKY TFs among the diverse signaling pathways and self-regulation is still not clearly understood.

The regulation and fine-tuning of WRKY TFs during plant stress responses contribute to the establishment of complex signaling networks and their crucial roles in plant AbS responses that make them potential candidates for imparting stress tolerance. More than 100 *WRKY* genes were predicted in rice [71]. They are upregulated in response to salinity, drought, and ABA, and downregulated in response to cold [71,72]. However, out of the extensive list of rice *WRKY* TFs studied, only a few genes have been functionally characterized with their response to AbS. The overexpression of *OsWRKY* genes, known to increase the sensitivity to cold and salt stresses [73], involved in the ABA signaling [12], enhanced drought and heat tolerance after heat pre-treatment as compared to wild-type plants [74]. When *OsWRKY* genes were over-expressed in Arabidopsis, besides an improvement of lateral root number and primary root length in the transgenic plants under osmotic stress, no clear phenotype regarding survival under AbS was shown [48,75] and increased sensitivity to ABA, salt, and osmotic stress [63]. The overexpression of *OsWRKY* TFs has induced higher sensitivity or enhanced stress tolerance, thus acting as both positive and negative regulators in stress signaling pathways [76].

This study is the foremost one to report an integrated genome-transcriptome-wide systematic analysis in C_3 model plant rice and also C_4 grass species. A deeper view of its importance in rice stress and systems biology particularly on AbS was investigated to identify and annotate the key players by computational omics approaches and examine their orthologs, differential expression signatures of spatio-temporal and plant hormones levels, interactome map, and molecular properties of the WRKY TFs in response to AbS. The systematic analysis provides insights for the molecular basis of *WRKY* TFs in *O. sativa* to stress responses, notably on plant AbS tolerance.

Based on the publicly available RNA-seq transcriptome data of *OsWRKY* genes, 26 out of 104 *OsWRKY* TFs showed a high and low level of hormonal expression. This expression signature data revealed that 26 potential *OsWRKY* genes for phytohormones such as ABA, JA, auxin, GA, CK, and BRs in the root and shoot of the rice plant at various time points. Thus, the obtained results revealed the lower expression of auxin and JA under field conditions. On the other hand, under a stressful environment, these two hormones are expressed in elevated levels and they play an important role in biotic and AbS conditions. From the heat map analysis, 26 potentially expressed AbSR *OsWRKY* TF genes were used for further functional analysis.

Spatio-temporal expression profiling of 26 *OsWRKY* genes showed the differential expression in 48 different tissue/organ-specific and developmental stages at individual abiotic stress conferring the higher expression level of *OsWRKY* genes from various tissue-specific and organs-specific expression dynamism under field conditions. This analysis revealed that *OsWRKY* TFs could be potential candidates for further functional characterization and distinct expression patterns for explaining their roles in AbS signaling. Further,

this data provides the support for conducting overexpression studies and metabolic engineering in different plant tissues in order to increase the number of AbSR gene content and also to enhance the nutrition content in rice. Moreover, the properties of the genes have many differences in M.Wt, amino acid length, aliphatic index, and pI of these genes which contain novel variants, which need to be delineated further for validation.

A phylogenetic tree of *OsWRKY* and WRKY TFs from C4 plants such as *SiWRKY*, *SbWRKY,* and *ZmWRKY* was constructed in accordance with the multiple sequence alignment of their corresponding WRKY domains. The position of WRKY DNA—binding domain (WRKY) and hAT family C-terminal dimerization domain (Dimer_Tnp_hAT) in *OsWRKY*, *SiWRKY*, *SbWRKY,* and *ZmWRKY* have been analyzed by HMMSCAN. This unrooted tree showed the distribution and divergence of *WRKY* domains and conserved regions present in candidate genes. Phylogenetic analysis showed that the predicted gene sequences highly diverge to *S. italica*, *S. bicolor*, and *Z. mays*. *OsWRKY* and their respective molecular cross-talks and functional relationships unraveled the complexity of unique and combined abiotic stress upon evolutionary seed gene modules and their connecting nodes, edges, and genes that were expressed in AbS studies [4,77].

Comparative mapping of *WRKY* and their respective genes on rice, sorghum, maize, and foxtail millet were performed to unveil the collinearity between the rice and C_4 grass species. *OsWRKY* showed maximum orthology of genes with *S. italica, Z. mays,* and *S. bicolor* owing to their wide range of chromosomal synteny. This analysis clearly shows that *OsWRKY* genes are highly similar to *SbWRKY*, *SiWRKY,* and *ZmWRKY* TFs and this close evolutionary relationship revealed the putative novel variants about C_3 and C_4 model crop plants, particularly grass species. This gene synteny information could pave the way for understanding the molecular evolutionary analysis and also could be used to conduct the over-expression and molecular breeding studies of *OsWRKY* genes among Poaceae members.

Comparing with rice (104 *WRKY*s), Arabidopsis (*Arabidopsis thaliana*) and wheat (*Triticum aestivum*) contains a proportionate number of WRKY genes (72 *AtWRKY*s and 171 *TaWRKY*s) [78,79], among which a certain number of genes play an important role in AbS. Drought is one of the most common AbS, that has a severe impact on crop growth and yield [80]. Rice *OsWRKY11* and *OsWRKY72* play an important role in drought tolerance [81,82] in analogous with Arabidopsis genes such as *AtWRKY57, AtWRKY63* [83,84], and wheat genes *TaWRKY14, TaWRKY90, TaWRKY8, TaWRKY122*, and *TaWRKY45* [85]. In addition, 12 *TaWRKY*s were recognized as the candidate drought-responsive genes, which are orthologous to genes in Arabidopsis and enhances during water deprivation [78]. Thus, the characterization of WRKYs in rice will help to unravels the AbS associated regulatory networks.

5. Conclusions

In this study, we have identified 26 *OsWRKY* genes that are responsible for various AbS via the computational systems biology approach. The gene properties, evolutionary analysis, gene structure, gene ontology annotation, and gene interaction networks of *OsWRKY* were evaluated. *OsWRKY* TFs and their spatio-temporal and phytohormonal expression of these candidate genes showed their differential expression signatures in various rice plant tissues and plant growth hormones, respectively. In addition to that, comparative mapping analysis exhibited that the maximum similarity with C_4 grass species such as *S. italica*, *S. bicolor*, and *Z. mays*. Thus, provides an important indication of their regulatory functions in AbS stress conditions. This study also provides depth information about *OsWRKY* TF genes and delineates the plant developmental metabolisms and their functional regulations under AbS conditions. This holistic study also hypothesizes that the identified candidate players may interact with various stress responsible TF family members and activates the transcriptional regulation, antioxidant enzymes, ROS scavenging mechanisms, biosynthesis of amino acids, cellular and physiological processes, and

synthesis of polyamines in response to AbS tolerance. Further functional analyses are needed to unravel the novel avenues of the identified players.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11071301/s1, Figure S1: Meta expression analysis of 104 *OsWRKY* TF genes, Table S1: WRKY name and NCBI ID in C3 and C4 grass plants; Table S2: *OsWRKY* genes and their HMMSCAN report; Table S3: *OsWRKY* genes and their attributes; Table S4: Orthologous relationship between *O. sativa* and *S. italica*; Table S5: Orthologous relationship between *O. sativa* and *S. bicolor*; Table S6: Orthologous relationship between *O. sativa* and *Z. mays*.

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Abbreviations

AbS—Abiotic Stress; CAbS—Combined Abiotic Stresses; *Os—Oryza sativa*; *OsWRKY—Oryza sativa WRKY*; *SbWRKY—Sorghum bicolor WRKY*; *SiWRKY—Setaria italica WRKY*; *ZmWRKY—Zea mays WRKY.*

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