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Research Article

Genome-wide Identification and Expression Analysis of WRKY Transcription Factor Gene Family in Date Palm (*Phoenix dactylifera*) Reveals its Role in Salt and Drought Stress Responses

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ABSTRACT

The WRKY transcription factors are pivotal in regulating several imperious plant growth transcriptional, biological, and metabolic processes. They are integrally involved in various stress responses in plants, significantly biotic and abiotic stresses. WRKY transcription family has been investigated in a large number of plant species, but their characterization and identification are not carried in date palm (*Phoenix dactylifera* L.) so far. The present study identified 66 non-redundant WRKY-encoding genes that were dispersed unevenly on all 16 chromosomes of the date palm. Based on phylogenetic analysis, PdWRKY genes were classified into three Groups (I, II, and III) while Group II was further divided into five sub-groups i.e., IIa, IIb, IIc, IId, and IIe. Intron-exon analysis exhibited the presence of 2-5 introns within all PdWRKY genes and a variable number of conserved motifs were observed in PdWRKY proteins. Moreover, the signatory WRKY domain was completely conserved, and many other domains were found to be conserved irregularly in PdWRKY proteins. More than fifty cis-acting regulatory elements were identified in upstream promoter regions. The transcriptomic data exhibited the expression patterns and variations of PdWRKY genes in shoot and root tissues stressed under drought and salt conditions. Interestingly, *PdWRKY49* and *PdWRKY58* showed similar responses in both shoot and root tissues under salt stress while in drought stress almost all PdWRKY genes upregulated in root tissues and showed downregulation in shoot tissues except *PdWRKY59* in both tissues respectively. Our findings demonstrated that WRKY proteins frequently act as repressors, along with as activators. Furthermore, the present study indicates that WRKY transcription factors could potentially orchestrate a diverse array of seemingly unrelated functions.

Keywords: WRKY gene family, WRKY transcription factors, *Phoenix dactylifera,* Phylogenetic analysis, Expression analysis, abiotic stresses, salt stress, drought

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INTRODUCTION

The date palm (*Phoenix dactylifera* L.) is a member of the Palmaceae family, that includes over 200 genera and more than 2500 species. Its name is derived from two Greek words "Phoenix" which means purple or red (fruit) and "dactylifera" which refers to the finger-like shape of the fruit clusters. Date palm trees are dioecious, meaning they have distinct male and female individuals (Chao and Krueger, 2007). Based on various historical accounts, the date palm was first cultivated over 6000 years ago in the Persian Gulf and subsequently introduced to North Africa. Nonetheless, the specifics of its history and the genetic contributions from its

ancestors to our contemporary date palm remain somewhat unclear. Date palm contains bioactive compounds that contribute to defense mechanisms and potential health benefits. Date fruits have a high content of sugars such as sucrose, fructose, and glucose, which can range from 65% to 80% depending on the variety and ripeness of the fruit. The average protein content has been noted to fall between 1.22 and 3.30%. (Al-Karmadi and Okhoh, 2024). Dates are rich in carbohydrates, contain 14 different fatty acids, and include 15 important salts and minerals, along with various proteins. Six vitamins, and a significant amount of dietary fiber (El-Sohaimy and Hafez, 2010). Egypt, Iran, Saudi Arabia, and Iraq are the main producers of date palms around the globe.

For several decades, the WRKY transcription factor (TF) gene family has been studied in various plant species. Potential functional and ecological investigations of this crucial gene family in higher plant species are supported by the valuable information developed through various analyses. Early in 1994, the first WRKY gene from sweet potatoes (*Ipomoea batatas*) was successfully cloned and reported (Ishiguro and Nakamura, 1994). Since then, advances in whole-genome sequencing have led to the identification of WRKY gene family in numerous plants including *A. thaliana*, which has variable WRKY members (Rushton *et al.*, 2010; Wang *et al.*, 2011), Rice (*Oryza sativa*) has 107 members (Wu *et al.*, 2005), soybean (*Glycine max*) 182 (Bencke-Malato *et al.*, 2014), maize (*Zea mays*) 125 (Hu *et al.*, 2021), pineapple (*Ananas comosus*) 54 (Xie *et al.*, 2018), chickpea (*Cicer arietinum*) 70 (Waqas *et al.*, 2019), tomato (*Solanum lycopersicum*) 81 (Huang *et al.*, 2014), rubber tree (*Hevea brasiliensis*) 81 (Li *et al.*, 2014) members, cotton (*Gossypium*) 116 (Dou *et al.*, 2014) and barley (*Hordeum vulgare* L.) contains 86 members (Zheng *et al.*, 2021). These WRKY transcription factors (IFs) are essential for many developmental and physiological processes. In addition to their role in the development of seeds and trichomes, panicle formation, leaf senescence, and floral bud differentiation, they also have a role in the regulation of plant responses to biotic and abiotic stresses (Ali *et al.*, 2014; Ali *et al.*,2018). They also mediate plant reactions to hormones, such as salicylic acid (SA) and jasmonic acid (JA) (Singh and Sharma, 2024). The WRKY domain consists of a conserved signature sequence WRKYGQK and a zinc-finger motif (Eulgem *et al.*, 2000). The WRKY family can be categorized into three groups (I, II, and III) based on the number of domains and characteristics of the zinc-finger motif. Both groups I and II have the C2H2 zine-finger motif. A CHC zinc finger motif is present in Group III along with a single WRKY domain (Eulgem *et al.*, 2000). With a characteristic zine-finger motif at the C-terminus, the domain of WRKY TFs is approximately 60 amino acids (aa) long at the N-terminus. It is reported that WRKY TFs can interact with the W-box (TTGACT/C) present in the promoter region of the genes involved in diverse processes ranging from the modulation of plant metabolism to biotic and abiotic stresses (Rushton *et al.*, 2010). Additionally, they play a key role in plant responses to pathogen-derived signals. Multiple W boxes are found in promoters of stress-responsive genes that often work together synergistically with the WRKY TFs. WRKY proteins specifically bind to these W boxes, a process confirmed through various binding studies (He *et al.*, 2019). WRKY proteins are key regulators in signaling pathways that activate defense-related genes, enhancing the resistance to bacterial and fungal pathogens, and making it essential for maintaining the health and productivity of economically important plant species. Therefore, it is imperative to understand the evolutionary origin of WRKY genes and duplications, as they are involved in vital plant processes and diverse regulatory functions. Understanding the evolutionary patterns of WRKY encoding genes in model plants like date palms will lead to comprehending their functional processes (Zhang and Wang, 2005).

The whole genome of date palm has been sequenced which facilitated molecular research on different aspects of its genome (Al-Mssallem *et al.*, 2013). However, in this study, all of the PdWRKY proteins from the genomic database were identified and subjected to phylogenetic, conserved domains, gene structure, and cis-regulatory element analyses. Here, we perform the detailed evolution and expansion of the WRKY TF family members in date palm and report their genome-wide identification and classification. Ortholog information from comparative phylogenetic and synteny analyses has also been used to propose the functions of discovered proteins. Furthermore, expression profiles of the discovered WRKY in distinct date palm tissues under salt and drought stress conditions are also studied. This research aims to understand the genomic, expressional, and functional patterns of PdWRKY genes and their response when stressed with various abiotic factors.

MATERIALS AND METHODS

Sequence retrieval and database search

All WRKY protein sequences of date palm were retrieved from the Plant Transcription Factor Database (PlantTFDB) https://planttfdb.gao-lab.org/family.php?fam=WRKY (Jin *et al.*, 2016). The physiochemical properties including Isoelectric points (PI), molecular weights (MW), and lengths of date palm genes were obtained through PlantTFDB. By using the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology and Information

(NCBI) (https://www.ncbi.nlm.nih.gov/), the putative WRKY protein sequences of date palm were validated and coding sequences (CDS) and genomic DNA sequences were also downloaded from NCBI. We also retrieved chromosomal positions and genomic information of date palm from NCBI.

Mapping of chromosomes, intron/exon distribution, and conserved protein motifs analyses

The positions of WRKY genes in the date palm genome were obtained from NCBI and the mapping of chromosomes was done by software named Mapchart (v.2.32) (Voorrips, 2002). Intron and exons distribution of PdWRKY genes was carried out by using Gene Structure Display Server (GSDS) v2.0 (https://gsds.gao-lab.org/) (Hu *et al.*, 2015) recently used by Shafique *et al.* (2023). Conserved protein motifs of PdWRKY proteins were visualized by MEME (Multiple Em for Motif Elicitation; v5.03) (https://meme-suite.org/meme/) (Bailey *et al.*, 2009).

Comparative phylogenetic and conserved domain analyses of WRKY proteins in date palm

The protein sequences of WRKY TFs from date palm (PdWRKY), *A. thaliana* (AtWRKY), *P. sativum* (PsWRKY), and *H. vulgare*as (HvWRKY) were retrieved from PlantTFDB and used to construct comparative phylogenetic tree. The nomenclature for protein sequences of PdWRKY genes was done according to Waqas *et al.* (2019), based on the positions of the genes on the chromosomes. For the construction of a phylogenetic tree, all the protein sequences from date palm and other reference plant species were subjected to multiple sequence alignment (MSA) through ClustalW (Thompson *et al.*, 2003; Ali *et al.*, 2024), employing default settings, the gap-opening penalties were consistently set at 10.00. However, the gap extension penalty was configured to 0.10 for pairwise alignments and 0.20 for multiple alignments. The negative matrix option was disabled and a delay divergence cutoff of 30% was applied. The resulting alignment helped to construct the phylogenetic tree by using the neighbor-joining (NJ) method with 1000 bootstrap replicates by using Molecular Evolutionary Genetics Analysis software (MEGA v11.0) (Tamura *et al.*, 2021). Further visualization and modifications were carried out by the webserver interactive tree of life (iTOL) v6.0 (https://itol.embl.de/) (Letunic and Bork, 2024). Different steps were carried out to identify the conserved domains with PdWRKY proteins. For this purpose, we used the NCBI conserved domain database (CDD) (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cg) (Marchler-Bauer *et al.*, 2015) to create a hit-data file and further visualization was done in TBtools-II software (Chen *et al.*, 2023).

Comparative synteny analysis of WRKY protein in date palm and Arabidopsis

The synteny analysis was performed by utilizing the Circoletto web server (https://bat.infspire.org/circoletto/) (Darzentas, 2010) to investigate the evolutionary relationships and similarities among the date palm (PdWRKY) and *Arabidopsis* (AtWRKY) WRKY proteins. Default settings were used for this purpose.

Cis-regulatory elements identification in the promoter region of WRKY genes

To identify the cis-regulatory elements in the promoters of date palm WRKY genes, a 1kb upstream promoter region was manually extracted from the NCBI for each PdWRKY gene. For the *in-silico* analysis of these promoter sequences to locate the cis-regulatory elements within the PdWRKY gene promoters, the PlantCare Database (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used (Lescot *et al.*, 2002). All data about the cis-regulatory elements were compiled and a tab-delimited file was used for each gene and was visualized in TBtools-II software (Chen *et al.*, 2023).

Use of RNA-seq data for expression profiling under abiotic stresses

The bio-projects PRJEB47833 (https://www.ncbi.nlm.nih.gov/search/all/?term=PRJEB47833) and PRJEB76162 (https://www.ncbi.nlm.nih.gov/search/all/?term=PRJEB76162) having 24 and 12 runs carrying and 4.3 Gb and 13.5 Gb of experimental data that were uploaded to the Sequence Read Archive (SRA) under accession numbers ERP132137 and ERP160713 respectively. Reference genome (NCBI RefSeq assembly: GCF_009389715.1) of date palm used to read the maps by using Galaxy Bowtie2 with default parameters (https://usegalaxy.org/) (De Almeida *et al.*, 2016). Experimental data were used to generate Fragments Per Kilobase per Million mapped fragments (FPKM) values through FeatureCounts, a Galaxy server-based tool (Liao *et al.*, 2013). FPKM values were calculated for all WRKY genes and were used to generate a heatmap by using TBtools-II software (Chen *et al.*, 2023).

RESULTS

Identification and characterization of WRKY genes in date palm genome

The complete genome of date palm allowed us to identify 78 genes that were predicted as WRKY genes. The presence of WRKY-specific primary domains in the encoding proteins of the selected genes was analyzed. The identification of a complete WRKY domain was the main parameter implemented to identify the WRKY proteins in the family. The 12 WRKY proteins were removed from consideration because, in their amino acid sequences, incomplete WRKY domains were observed. These proteins likely lost part of their functional domains during the evolutionary process, which led to

Table 1. Properties of identified *Phoenix dactylifera* WRKY genes and proteins.

TF ID	Given Names	Protein length	MW (Da)	PI	Gene Symbol	Chr. No.	Gene Start	Gene End	Exon Count
PDK_30s1011261g001	*PdWRKY45*	204aa	22863.7	5.1242	LOC103709614	9	21223814	21225222	3
PDK_30s1025361g003	*PdWRKY08*	575aa	63083.1	8.2245	LOC103717512	3	4286295	4289386	5
PDK_30s1051061g001	*PdWRKY12*	416aa	45570.4	6.375	LOC103702225	3	5128421	5130908	2
PDK_30s1051061g002	*PdWRKY11*	283aa	31037.8	5.045	LOC103702224	3	5115011	5119080	3
PDK_30s1053181g004	*PdWRKY58*	589aa	64856.8	9.1345	LOC103696297	12	6184087	6188759	8
PDK_30s1054411g002	*PdWRKY01*	254aa	28459.2	7.4324	LOC103723396	1	4284574	4287626	3
PDK_30s1065261g003	*PdWRKY29*	378aa	41947.5	9.1484	LOC103696592	7	10556090	10566970	4
PDK_30s1067021g003	*PdWRKY44*	171aa	18804.1	8.8727	LOC103709613	9	21207521	21209025	3
PDK_30s1078511g002	*PdWRKY61*	185aa	20290.3	4.8252	LOC103698294	13	8753756	8755699	2
PDK_30s1086261g001	*PdWRKY41*	280aa	30479.3	9.9088	LOC103712469	9	17460118	17462011	3
PDK_30s1093591g002	*PdWRKY49*	362aa	40301.7	6.7906	LOC103708338	11	1460107	1462876	3
PDK_30s1103751g023	*PdWRKY05*	469aa	51016	7.8854	LOC120104087	1	40285108	40290287	6
PDK_30s1138471g005	*PdWRKY52*	225aa	25270.8	7.4091	LOC103720433	11	2945908	2949138	4
PDK_30s1138471g008	*PdWRKY51*	149aa	15902.4	4.6011	LOC103721580	11	2924914	2926462	3
PDK_30s1149101g001	*PdWRKY18*	341aa	38153.3	7.861	LOC103697157	4	12090004	12092142	3
PDK_30s1158601g004	*PdWRKY23*	312aa	34092	10.1225	LOC103714091	7	389816	392154	3
PDK_30s1171101g004	*PdWRKY33*	106aa	12007.5	10.6563	LOC103710925	8	17587157	17589371	2
PDK_30s1173121g001	*PdWRKY28*	232aa	25364.5	9.12	LOC103707788	7	10277262	10279420	5
PDK_30s1175051g002	*PdWRKY40*	527aa	56540	6.6338	LOC103699521	9	15932154	15935769	6
PDK_30s1200411g002	*PdWRKY30*	353aa	39359.8	10.531	LOC103716572	8	12886893	12890605	3
PDK_30s1203911g001	*PdWRKY46*	370aa	40891.2	7.4442	LOC103705538	9	21265107	21271252	6
PDK_30s65509418g001	*PdWRKY14*	298aa	33296.4	5.0912	LOC103706248	3	19274531	19276594	3
PDK_30s6550959g004	*PdWRKY38*	172aa	19110.5	8.7672	LOC103720801	8	21859444	21860857	3
PDK_30s6550959g005	*PdWRKY37*	211aa	23178.3	7.6333	LOC103720799	8	21849949	21851404	3
PDK_30s65509720g004	*PdWRKY20*	384aa	43264.3	4.8163	LOC103698036	6	9217829	9219916	5
PDK_30s65509724g007	*PdWRKY65*	327aa	36540.4	10.3398	LOC103718774	16	3654656	3658054	5
PDK_30s655861g002	*PdWRKY56*	447aa	49125	7.9967	LOC103708865	12	5217200	5220331	5
PDK_30s655861g003	*PdWRKY57*	291aa	32541.7	9.1961	LOC103708865	12	5217200	5220331	5
PDK_30s663661g001	*PdWRKY35*	243aa	27177.7	10.0553	LOC103709750	8	21045738	21051110	3
PDK_30s665281g005	*PdWRKY53*	282aa	32157.2	7.8825	LOC103720653	11	3229040	3234499	3
PDK_30s680531g012	*PdWRKY06*	302aa	33013.2	9.5499	LOC103706394	2	5083151	5087145	3
PDK_30s681021g002	*PdWRKY32*	475aa	51472.2	7.5335	LOC103703339	8	14850874	14853414	6
PDK_30s684781g004	*PDWRKY66*	254aa	28627.6	9.6791	LOC103700856	16	8549561	8557782	4
PDK_30s689431g003	*PdWRKY39*	117aa	13034.8	10.2696	LOC103720801	8	21859444	21860857	3
PDK_30s691921g002	*PdWRKY60*	262aa	28643.4	9.316	LOC103696886	13	7439682	7441248	3
PDK_30s692531g003	*PdWRKY07*	327aa	36307.4	4.8741	LOC103697265	3	963038	968437	3
PDK_30s705791g001	*PdWRKY50*	175aa	19957.5	8.5729	LOC120112317	11	2906219	2908669	3
PDK_30s716811g001	*PdWRKY27*	229aa	24902.5	10.605	LOC103707065	7	5434290	5435871	3
PDK_30s722152g002	*PdWRKY55*	324aa	36271.4	8.428	LOC103713231	11	11179710	11179710	3
PDK_30s742801g002	*PdWRKY62*	583aa	63893.2	6.9228	LOC103721507	13	9258081	9261073	5
PDK_30s745031g002	*PdWRKY34*	359aa	39969.2	5.4493	LOC103702950	8	18496062	18501944	3
PDK_30s771211g003	*PdWRKY21*	559aa	61637.8	7.3304	LOC103714938	6	11784290	11795752	8
PDK_30s777581g001	*PdWRKY48*	532aa	58715.8	6.2591	LOC103713914	10	11623067	11676463	6
PDK_30s785891g002	*PdWRKY17*	358aa	39898.2	6.0539	LOC103695902	4	4260793	4263346	3
PDK_30s806661g003	*PdWRKY64*	297aa	32509.7	6.7961	LOC103707403	15	1903543	1918739	7
PDK_30s816181g001	*PdWRKY10*	160aa	18318.8	6.5083	LOC120110172	3	5108491	5110625	3
PDK_30s816181g002	*PdWRKY09*	306aa	33708.7	5.9139	LOC103702223	3	5102035	5104627	3
PDK_30s816181g003	*PdWRKY13*	289aa	31775.5	7.1502	LOC120110335	3	5463510	5465803	3
PDK_30s835131g001	*PdWRKY47*	182aa	20981.5	9.9454	LOC103720275	10	8418427	8421246	2
PDK_30s839411g020	*PdWRKY26*	253aa	28525	6.8515	LOC103710421	7	2286053	2289643	4
PDK_30s839411g021	*PdWRKY25*	289aa	32175.1	7.9122	LOC103710422	7	2276980	2278745	5
PDK_30s839411g024	*PdWRKY24*	453aa	50169.5	8.6249	LOC103710430	7	2205250	2216164	5
PDK_30s842671g002	*PdWRKY31*	353aa	39650.1	10.3289	LOC103716572	8	12886893	12890605	3

PDK_30s849681g002	*PdWRKY03*	291aa	32066.7	7.9891	LOC103698690	1	28814889	28829154	4
PDK_30s853591g002	*PdWRKY36*	515aa	55286.2	8.253	LOC103721327	8	21705322	21710683	6
PDK_30s868971g001	*PdWRKY43*	75aa	8106.1	11.0209	LOC103713754	9	19324657	19326223	3
PDK_30s880731g006	*PdWRKY15*	401aa	43845.1	9.2471	LOC103702844	3	21731302	21736384	6
PDK_30s888801g002	*PdWRKY02*	695aa	75983.5	7.2282	LOC103710681	1	13753223	13758109	5
PDK_30s904851g001	*PdWRKY19*	239aa	26587.6	9.5597	LOC103699722	5	602647	606833	3
PDK_30s912911g001	*PdWRKY16*	222aa	24977.4	8.3687	LOC103704194	3	23095771	23098486	3
PDK_30s914091g001	*PdWRKY22*	361aa	39304.5	7.1959	LOC103705233	6	14341585	14358149	7
PDK_30s930251g004	*PdWRKY59*	288aa	32397.4	5.8797	LOC103697177	12	7071054	7073589	3
PDK_30s932191g002	*PdWRKY42*	364aa	40576.1	7.6445	LOC103708157	9	17986913	7990629	3
PDK_30s936891g001	*PdWRKY04*	315aa	35120.6	8.8919	LOC103716221	1	29156973	29158781	5
PDK_30s969751g001	*PdWRKY63*	116aa	13662.5	10.295	LOC103721211	14	4183177	4187713	2
PDK_30s975231g001	*PdWRKY54*	577aa	61900.9	6.2849	LOC103715016	11	10767467	10769239	4

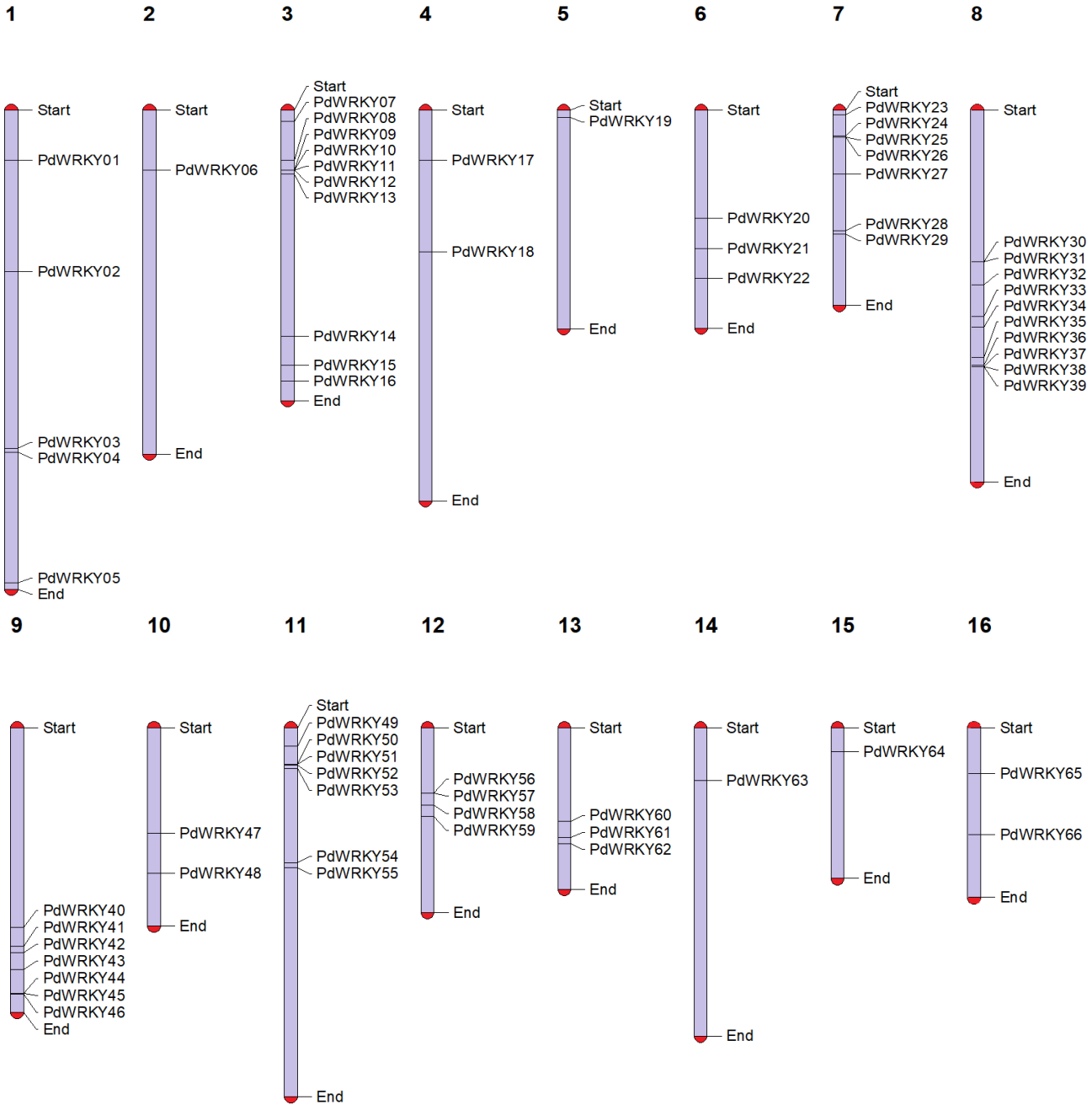

Figure 1. Chromosomal distribution of WRKY genes in date palm. The black lines on chromosomes represent the distributed locations of WRKY genes. The red-colored segments show the start and end points of chromosomes.

incomplete or duplicated amino acid sequences. These genes thus count as redundant and are often referred to as pseudogenes, as has been observed in several other species (Panchy *et al.*, 2016; Waqas *et al.*, 2019). By following the ascending order of the remaining 66 non-redundant PdWRKY genes on their respective chromosomes their nomenclature was developed (*PdWRKY1* to *PdWRKY66*) as described by Eulgem *et al.* 2000, Li *et al.* (2015), Waqas *et al.* (2019), Shafique *et al.* (2023) and Anas *et al.* (2023). Peptide lengths of PdWRKY proteins varied from 75 to 695 amino acids carrying an average of 323.2 amino acids. The isoelectric point (pI) values ranged from 4.60 to 11.02 while molecular weight was ranging from 8106.1 to 75983.5 Da, bearing the average of 35479.8 Da in PdWRKYs (Table 1).

Genomic localization of WRKY family encoding genes in date palm

The identified PdWRKY genes were distributed on all 16 chromosomes (Figure 1). The maximum number of PdWRKY genes (10) were distributed on chromosomes three and eight each. There were 7 PdWRKY genes on chromosomes seven, nine and eleven each. Five genes were localized on chromosomes one, four and twelve, while only three genes were observed on chromosomes six and thirteen. Chromosomes four, ten and sixteen each were carrying only 2 PdWRKY genes. The remaining chromosomes (two, five, fourteen and fifteen) each exhibited only one PdWRKY gene. Most PdWRKY genes were clustered on the chromosomes, signifying they may constitute a single QTL (Quantitative Trait Loci) (Waqas *et al.*, 2019).

Mostly PdWRKY genes were involved in different molecular functions like, stress response, nuclear localization within cellular components, transcriptional regulation and DNA binding. WRKY transcription factors respond in multiple plant species when exposed to different abiotic stresses like heat, cold, salinity, senescence, dark, wounding, UV and carbon famine (Chen *et al.*, 2019). According to our study, all PdWRKY genes were involved in defense mechanisms such as biotic stresses, transcription factor activities, sequence-specific DNA binding and DNA-templated activities. Interestingly, at the molecular level *PdWRKY04* gene was involved in exhibiting the promoter-specific chromatin binding and biologically was involved in responsiveness of different metabolic processes i.e., gibberellin, ethylene, cold, water deprivation, salt stress, and parasitic fungus. Moreover, *PdWRKY64* gene was biologically involved in lateral root development, atrichoblast differentiation, negative transcription regulation from the RNA polymerase II promoter, and response to nutrient levels and was also involved in DNA binding specific to core promoter sequences at the molecular level Specifically, *PdWRKY13* gene responded to chitin, systemic acquired resistance, induced systemic resistance and signaling pathways in particular to jasmonic acid and salicylic acid. *PdWRKY41* and 35 were specifically involved in leaf senescence and regulation of lignin biosynthetic process respectively (Table S1). Our results concluded that PdWRKY genes play a crucial role in various plant processes, like regulating seed dormancy and signaling pathways, and act as activators that influence the expression of other genes and contribute to the overall plant development and response mechanisms. All molecular and biological functions of all PdWRKYs are given in Supplementary Table 1.

Comparative phylogeny of WRKY transcription factors in *P. dactylifera*, *A. thaliana*, *P. sativum* and *H. vulgare* and conserved domain analyses

To investigate the evolutionary relationship, we performed a phylogenetic analysis combining 307 putative from PdWRKY, AtWRKY, PsWRKY, and HvWRKY genes to create an unrooted neighbor-joining (NJ) tree (Figure 2). The tree was divided into three groups of orthologous genes (I, II, and III). Group II was the largest group with a total of 169 WRKY genes from all four species and was further classified into five sub-groups IIa, IIb, IIc, IId, and IIe with 37, 16, 57, 24 and 7 genes respectively. There were 76 and 62 WRKY genes present in Group I and III respectively. As the number of genes was different in all groups, so presence of orthologs in all groups was unexpectedly varied. Sixty-five orthologs were present in the whole of Group II with 9 in Group II, 13 in Group-IIa, 4 in Group-IIb, 23 in Group-IIc, 12 in Group-IId and 4 in Group-IIe. Individually, Group III had a maximum (25) number of orthologs, and 20 orthologs were present in Group I. Conserved domains were predicted in all PdWRKY proteins using NCBI CDD and were visualized in Tbtools-II software (Figure 3). Six conserved domains WRKY, WRKY Superfamily, Plant-Zn-Clust, Plant-Zn-Clust superfamily, PRK11901 and PRK11901 Superfamily domains were identified in all peptide sequences of PdWRKY. WRKY domain was conserved in all PdWRKY proteins excluding *PdWRKY11*, *39,* and *51* and these proteins contained WRKY superfamily domain. Plant-Zn-Clust superfamily domain was also observed in PdWRKY23, *27*, *30*, *31,* and *65* proteins along WRKY domain. Interestingly, PRK11901 Superfamily domain was found only in *PdWRKY40* protein with WRKY domain.

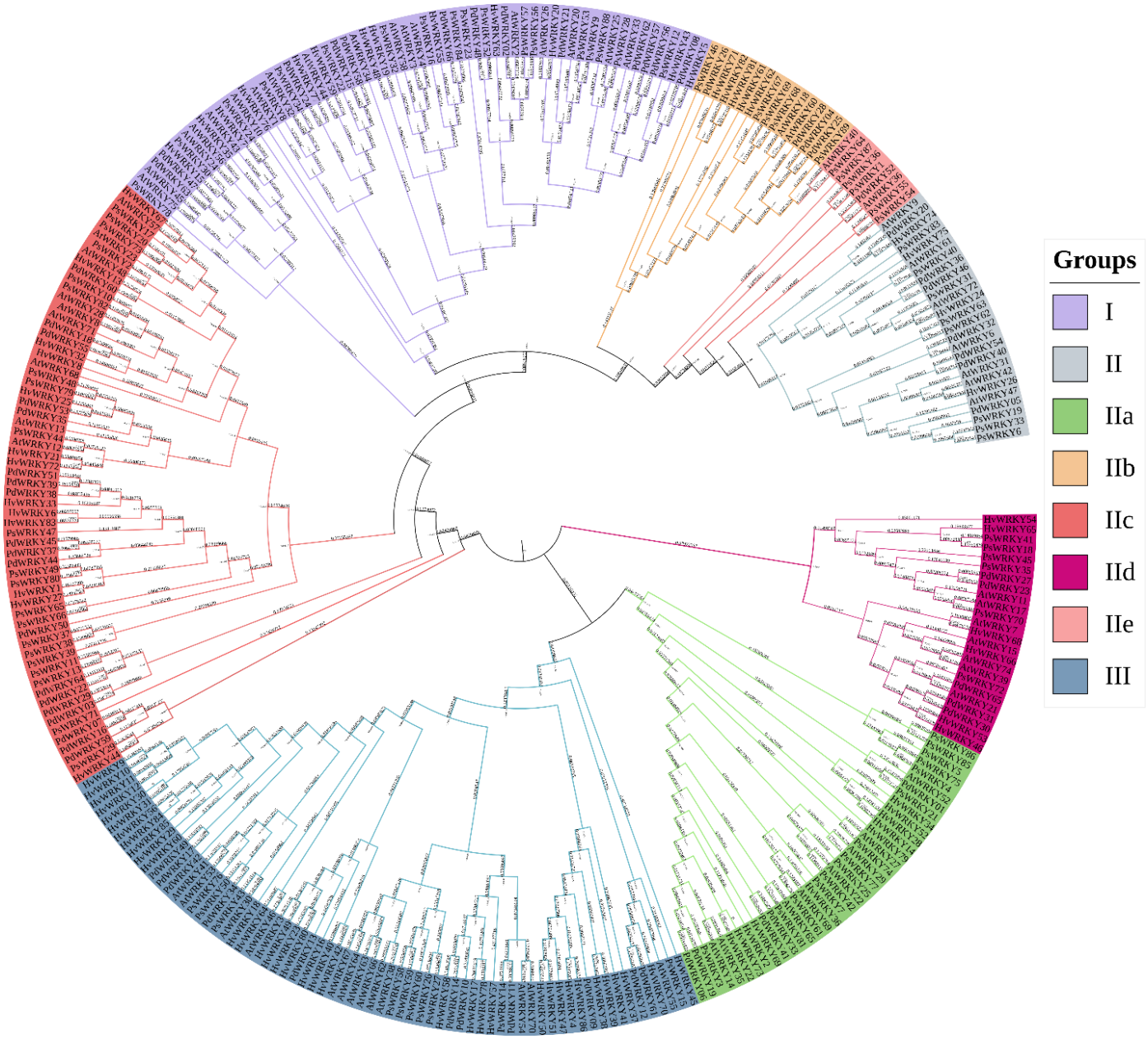

Figure 2. Comparative phylogenetic tree of WRKY proteins from Date palm, *A. thaliana*, *P. sativum* and *H. vulgare*. The full-length amino acid sequences of PdWRKY, AtWRKY, HvWRKY and PsWRKY, were aligned by ClustalW and a rooted phylogenetic tree was computed by MEGA v.11 software using the neighbor-joining (NJ) method. Different major clusters of orthologous genes, as shown in the legend, are distinguished by different colors.

Gene structure and conserved motifs analyses:

The distribution of introns and exons had been analyzed to gain further insights in the evaluation of the WRKY gene family in date palm. In scrutinizing 66 WRKY genes of date palm, we identified the positions of introns and exons within their genomic and coding sequences (Figure 4). PdWRKY genes exhibited a diversity concerning the variation among number of introns e.g. ranges from 01 to 05. The analysis revealed the presence of only two introns in most of the PdWRKY genes, while in *PdWRKY15*, 02, 21, 46, 36, 05, 40, and 32 genes maximum number of introns (five) were observed. However, PdWRKY58, 08, 62, 57, 04, 28, and 46, *PdWRKY24*, 66, 48, 29, 03, 64, 22, 26, 52 and 54 and *PdWRKY01*, 12, 47, 63 and 61 genes had four, three and two introns respectively. There was no UTR observed at the 3' and 5' ends of the *PdWRKY11* and 12 genes. Exceptionally, in *PdWRKY13* gene UTR was present at the 3' end only.

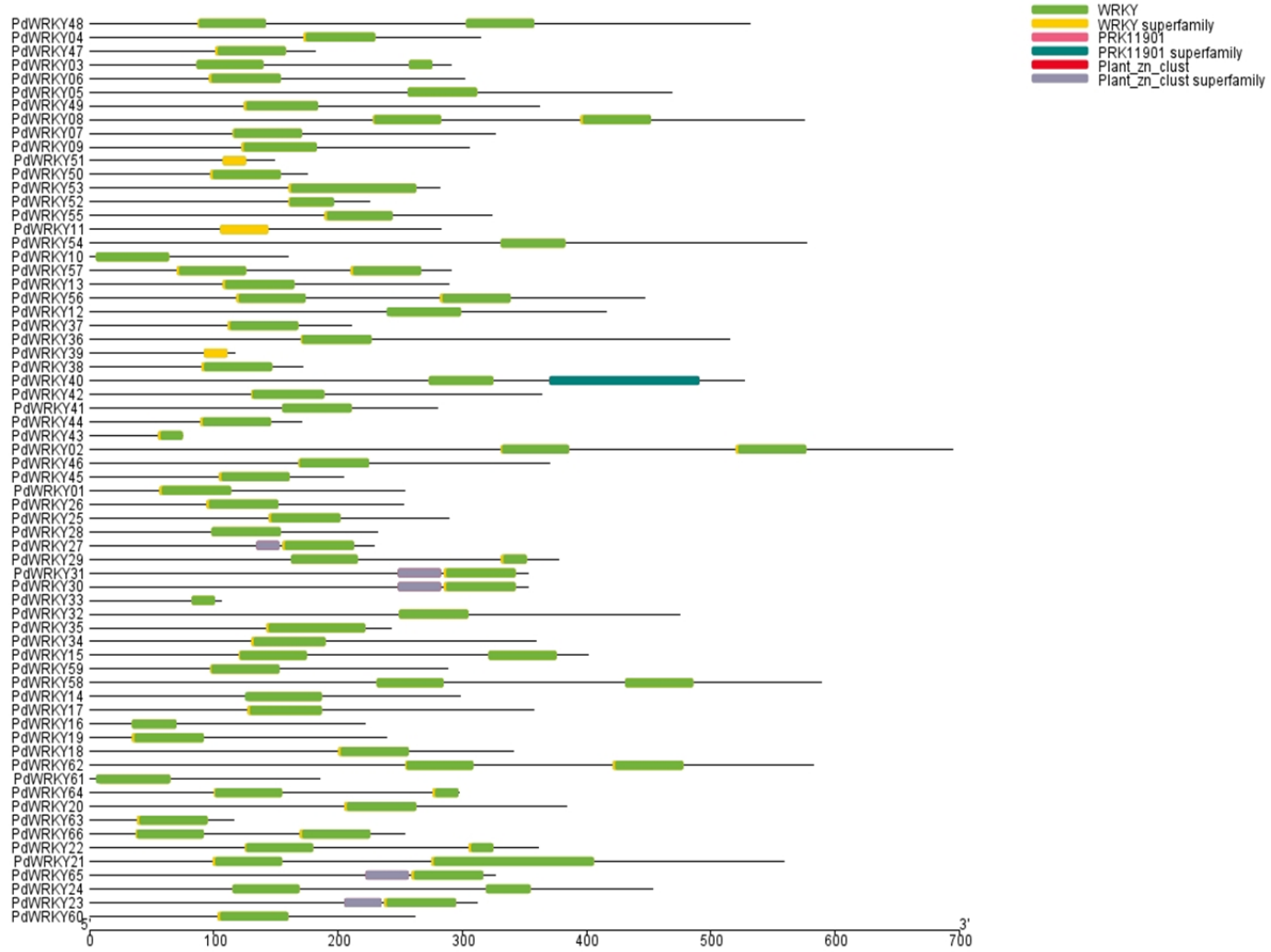

Figure 3. Conserved domain regions of PdWRKY genes green, yellow, pink and zinc colors conserved domains represent WRKY, WRKY superfamily, PRK11091 and PRK11091 superfamily respectively.

Table 2. Different attributes of motifs discovered by using MEME suite.

No. of motif	Motif Sequences	E- value	Sites	Width
1	WRKYGQKVVKGSPYPRSYYRC	3.0e-1019	63	21
2	TPGCPVKKQVZRSSEDPSIVITTYEGKHN	2.7e-734	48	29
3	STSQEKTVREPRVAVQTRSEVDILDDGYR	3.8e-321	34	29
4	DGYNWRKYGQKQVKGSENPRSYYKCTHPNCPVKKKVERSLDGQITEIVYK	7.6e-298	9	50
5	LNRMREENKRLKTMLAQITKBYQSLQMHFFDJMQZRAQKKG	3.7e-084	11	41
6	DGSILIITYEGZHNH	5.9e-063	14	15
7	ARCSPRSEBSSGAFATAGRCECSKKRKTLVKRSIQVPAISGKLAEGPLDE	7.8e-086	8	50
8	PLPPAATAMASTTSAAASMLLSGSMSST	3.5e-043	6	28
9	WRKYGQKVVKGNPNP	1.4e-041	11	15
10	MAYFPSERSDWRAIAMSMAFGHHSGWPRVMNASDYMLYDEASAACQLE	5.9e-039	3	48

The most conserved 21-residue-long motif (WRKYGQKVVKGSPYPRSYYRC) motif1 was observed in every PdWRKY protein except PdWRKY11, 16, and 43 (Figure 5). Only a single conserved motif1, 9 and 2 were present in *PdWRKY10*, 11, 16 and 43 proteins respectively., The longest motif was Motif 4 with a 50-residue-long (DGYNWRKYGQKQVKGSENPRSYYKCTHPNCPVKKKVERSLDGQITEIVYK) sequence and motif4 sequence with least number of residues (DGSILIITYEGZHNH). These conserved motifs are responsible for the distinct roles of the PdWRKY genes in various cellular and biological processes. Different attributes of all ten motifs are given in Table 2.

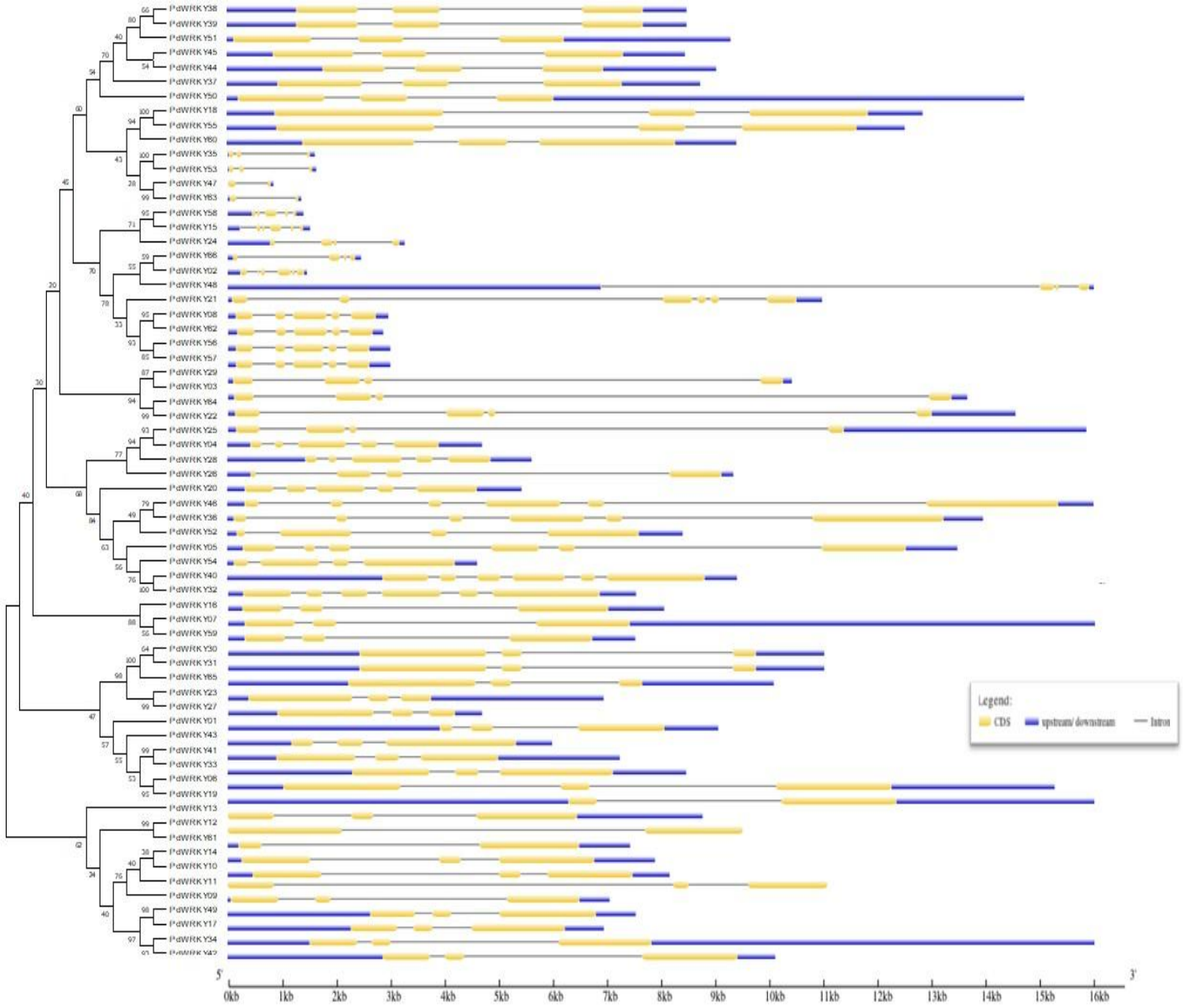

Figure 4. Phylogenetic tree-based clustering and structure of PdWRKY genes. We created an un-rooted phylogenetic tree based on the protein sequences of PdWRKY with 1000 bootstrap replicates. The yellow, blue, and grey colors represent CDS, up/down streams, and introns respectively.

Promoter analysis of cis-acting regulatory elements of WRKY genes and synteny analysis

Cis-acting regulatory elements are insulators, inhibitors and enhancers which regulate the gene expression levels present in promoter regions (Rombauts *et al.*, 1999; Rombauts *et al.*, 2003). Cis-acting regulatory elements also known as molecular regulators which impact the gene transcription and a range of biological processes, such as hormones, stress response and developmental processes (Hernandez-Garcia and Finer, 2014). Therefore, various conserved cis-acting regulatory elements like ABRE involved in seed specific regulation, LTR low temperature responsiveness, MBS elements related to drought-inducibility, TATC-box and p-box gibberellin-responsive element, ARE cis-acting regulatory element essential for the anaerobic induction and CAAT-box involved in association of enhancer and promoter regions of common *cis*-acting elements were identified repeatedly in PdWRKY genes and were involved in their specific gene regulatory functions (Figure 6). Moreover, multiple cis-acting regulatory elements like GATA-motif, TCT-motif, GT-1 motif, ACE, I-box and G-Box were involved as light responsive elements in frequent PdWRKYs. This information evidents the involvement of PdWRKY genes in various stress responses. All information about remaining cis-acting regulatory elements and their specific roles in regulation of gene functions is given in Table S1.

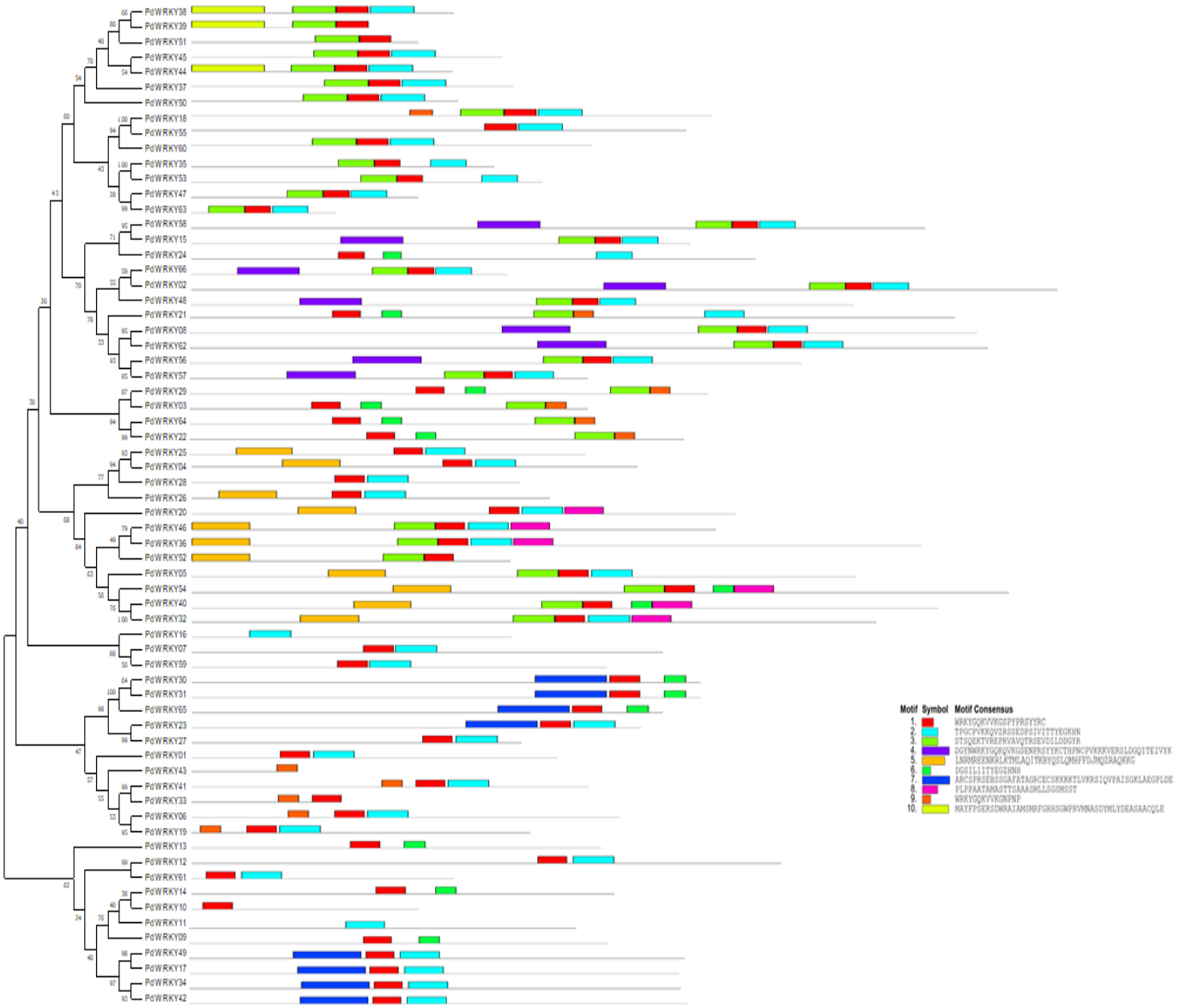

Figure 5. Conserved motifs and Phylogenetic tree-based clustering of PdWRKYs. Colors are representing the motifs at near bottom of the figure. Longer the sequence significantly more conserved the motif then short sequence motif.

Synteny analysis

The conservation relationship among the genes the of WRKY TFs of date palm and *A. thaliana* was exhibited. To create the ideogram AtWRKY genes were used as reference against PdWRKY genes (Darzentas, 2010). The syntenic relationship with 100% conservation was observed between *PdWRKY54* with *AtWRKY31*, *42*, *PdWRKY32* with *AtWRKY42*, *PdWRKY2* with *AtWRKY2*, *PdWRKY40* with *AtWRKY42*, *PdWRKY62* with *AtWRKY33*, *PdWRKY36* with *AtWRKY72*, *PdWRKY8* with *AtWRKY33* and *PdWRKY22* with *AtWRKY3* representing in red color of Figure 7. Interestingly, all the genes sharing conserved syntenic regions were involved in the transcriptional regulatory region DNA binding and sequence-specific DNA binding functions.

Gene expression pattern of WRKY transcription factors in roots and shoots of date palm under salt and drought stresses

Identifying and characterizing the PdWRKY genes helps us to understand the molecular mechanisms that help the date palm to tolerate salinity and drought stresses (Yaish *et al.*, 2017; Ali-Dinar *et al.*, 2023). Salt-enriched rhizosphere lowers the transpiration, stomatal conductance, and CO_2 assimilation in date palm (Ramoliya and Pandey, 2003; Pongrac *et al.*, 2024). However, plants use various strategies to deal with drought stress conditions. Plants alter the hormones, solutes, and carbohydrates responses to deal with drought conditions (Ali-Dinar *et al.*, 2023). By following these patterns, plants develop remarkable resilience and the ability to survive in water-scarce environments (Torres-Franklin *et al.*, 2007; Iqbal *et al.*, 2024).

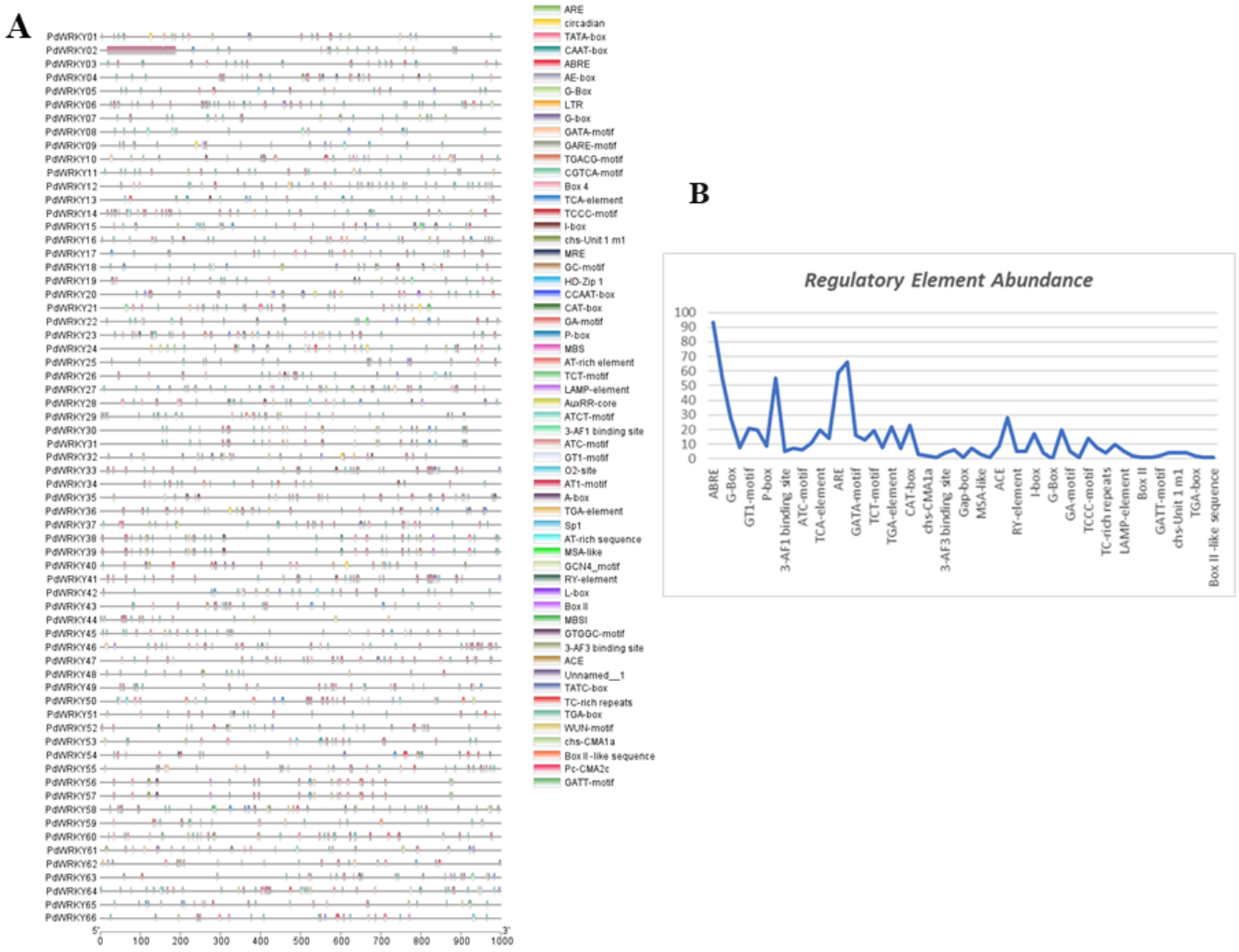

Figure 6. Prediction of cis-regulatory elements, (A) Cis Acting Regulatory Elements of PdWRKY genes are visualized in the figure and organized genes are as well while in the ligand figure colors of cis-acting regulatory elements are shown. (B) The regulatory Elements abundance graph shows that ABRE regulatory elements are most abundantly present in PdWRKY gens, P-Box, and ARE regulatory elements are also in abundance.

We used the RNA-Seq bio-projects as mentioned in the Material and Methods section, where root and shoot tissues of Khalas (a salt and drought tolerant date palm variety) were stressed with NaCl and drought conditions, and their RNA-seq data were compared with control. In salt-stressed conditions, about all the PdWRKY genes in root tissues were upregulated while *PdWRKY12*, *15,* and *16* showed downregulation (Figure 8a). However, *PdWRKY37*, *50*, *59* and *61* didn't show any specific response when stressed with NaCl in root tissues. Interestingly, most of PdWRKY genes in shoot tissues stressed with NaCl were downregulated except *PdWRKY16* and *37* which were significantly upregulated. On the other hand, in drought stress conditions all the PdWRKY genes in root tissues were significantly upregulated except *PdWRKY59* which gave no kind of specific regulation. While opposite results were observed in shoot tissues from which most of the PdWRKY genes were downregulated. However, *PdWRKY49* and *59* genes showed significant upregulation in similar drought conditions. The results of both experiments were compared with control parameters.

DISCUSSION

One of the most ancient plants, *Phoenix dactylifera* (date palm) has been under cultivation for thousands of years in North Africa and the Middle East. It contains proteins, carbohydrates, vitamins, minerals, and dietary fibers in rich quantities (Alotaibi *et al.*, 2023). Like other crops, salinity and drought stresses adversely affect the growth and production of date palm (Alhammadi *et al.*, 2012). Studies revealed the lack of data availability in response to salt and drought stress in WRKY transcription factor of Date palm. WRKY TF family which regulates the developmental processes of plants but also modulates the various stress responses in plants (Rushton *et al.*, 2010).

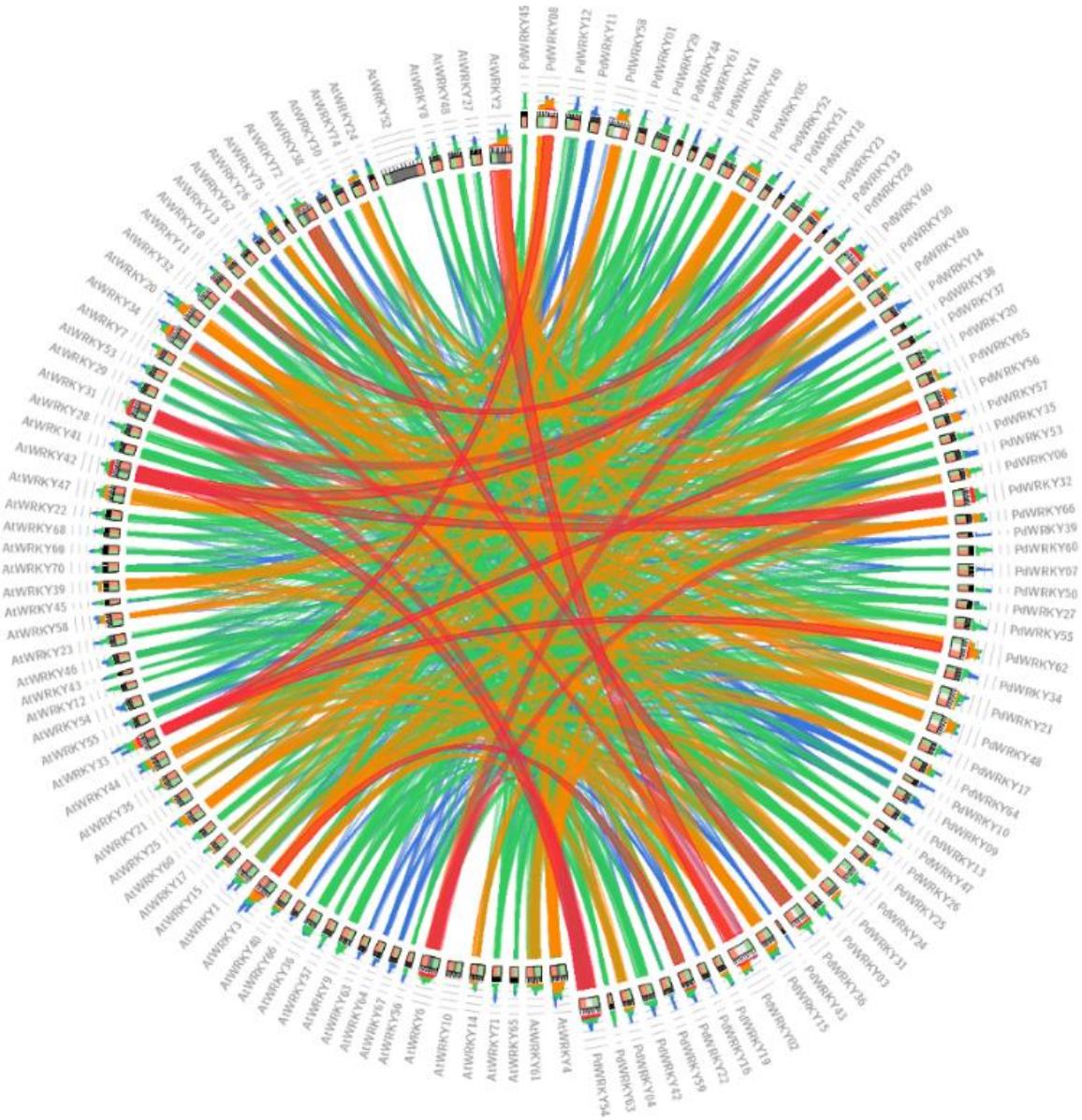

Figure 7: The syntenic regions in selected PdWRKY proteins by taking the AtWRKY proteins as the query sequence. blue, green, orange, and red are 50, 75, 99.99, and 100% similar, respectively.

In the present study, we report 66 non-redundant WRKY genes in date palm. Compared to other species of plants, date palm carries greater WRKY genes number i.e., rapeseed (43) (Yang *et al.*, 2009), barley (45) (Mangelsen *et al.*, 2008), cucumber (55) (Ling *et al.*, 2011), castor bean (58) (Zou *et al.*, 2016) and grape wine (60) (Wang *et al.*, 2014). However, other plant species which reportedly contain a greater number of WRKY genes than date palm are tomato (78) (Chen *et al.*, 2015), rice (100) (Wu *et al.*, 2005), cotton (120) (Cai *et al.*, 2014), soybean (131) (Yu *et al.*, 2016) and maize (136) (Wei *et al.*, 2012).

Comparative phylogeny functional adaptations and expression analyses

Previous studies and identifications of WRKY gene sequences in different plant species categorized all gene members into three major groups (Wu *et al.*, 2005; Zhang *et al.*, 2019; Khuman *et al.*, 2020). The phylogeny results of the current study based on comparative evaluation among WRKY gene species also revealed the three groups i.e., Group I, II, and III while Group II was further divided into five sub-groups (IIa, b, c, d, and e). Our results are consistent with previous studies and findings of *Arabidopsis* (Eulgem *et al.*, 2000) and cassava (Wei *et al.*, 2016). However, in date palm Group II presented the most PdWRKY genes with the maximum number of orthologs same as in the previous study by Waqas *et al.* (2019). The presence of both AtWRKY and PdWRKY was observed in each clade, which resulted from the conserved evolution of WRKY genes between these plants, and genes that form orthologous pairs commonly withhold their functions after convergence (Blanc and Wolfe, 2004).

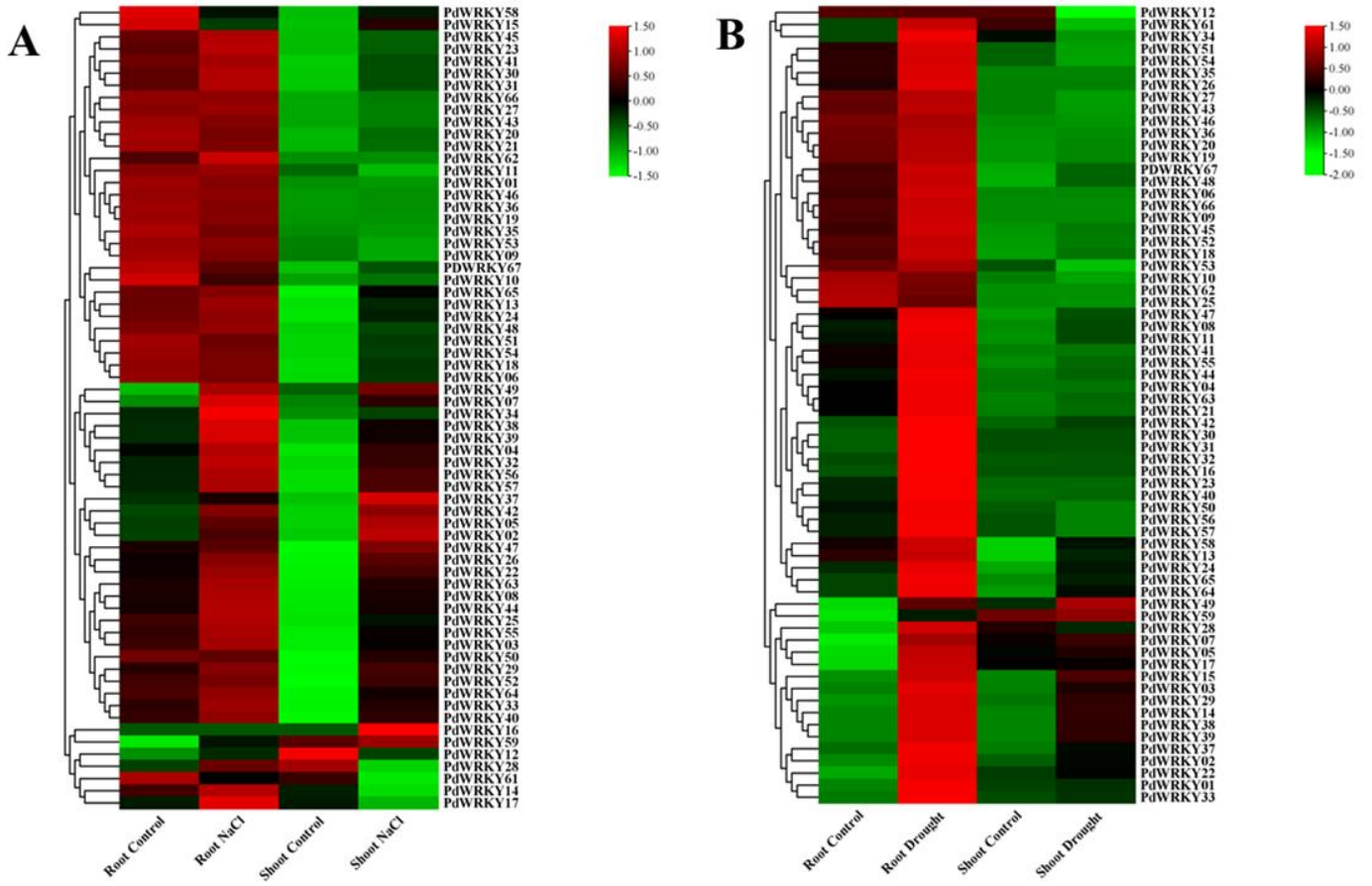

Figure 8: Transcriptomic analysis, the expression patterns of PdWRKY genes across different tissues of Date palm. A) PdWRKY gene expression under salt stress in Date palm. B) PdWRKY gene expression under drought stress in Date palm is visualized in a TBtools-II generated heatmap. Rows represent genes while columns control or stress experiments and colors indicate changes in expression levels.

For instance, in Group-IIa, *PdWRKY01* which is orthologous to *AtWRKY27*, and the latter is reported to be involved in responses to *Ralstonia solanacearum*, the causal organism of wilt diseases infection and ABRE cis-acting regulatory element involved in the abscisic acid responses (Mukhtar *et al.*, 2008; Narusaka *et al.*, 2003). However, in Group-IIb, *PdWRKY36* and *46* were orthologs of *AtWRKY72*. They could be possibly involved in defense against nematode and in response to drought stress by regulating MBS levels which are the functions of *AtWRKY72* (Yu *et al.*, 2001; Dong *et al.*, 2003; Kranz *et al.*, 1998). In *A. thaliana AtWRKY40*, an ortholog of date palm PdWRKY04 and 25 also showed upregulated the transcriptional level under salt treatment (Zou *et al.*, 2016) and this is in accordance with our results. Similarly, in Group-IIc, PdWRKY36 was also highly upregulated in response to both drought and salt stresses, and its ortholog *AtWRKY75* is involved in the modulation in of phosphate starvation in Arabidopsis (Devaiah *et al.*, 2007). Identifying the orthologs of AtWRKY in date palm could help in the validation of functional divergence of WRKY transcriptions in date palm.

Expression analysis based on transcriptomic data revealed the differential expression in PdWRKYs orthologs under salt and drought stresses in roots and shoots. Salt-affected soil causes disturbance in nutrient transportation, reduction in photosynthesis, ion toxicity, and osmotic adjustment (Al-Khateeb *et al.*, 2020). Similarly, drought stress also disturbed the nutritional, morphological, and physiological traits by affecting stomatal conductance, leaf water potential, photosynthetic pigment, and nitrogen and phosphorus absorption (Jaleel *et al.*, 2009; Baslam and Goicoechea, 2012). Both roots and shoots under salt stress had shown distinctive regulation of genes. In roots differently expressed genes showed significant upregulation (Yaish *et al.*, 2017) while in shoots most of the genes were downregulated with exception (Ait-El-Mokhtar *et al.*, 2019). WRKY TFs regulate the osmotic balance and significantly affect the expression level of various stress-related genes (Agarwal *et al.*, 2011). Our results demonstrated similarity with Tripathi *et al.* (2014), most of the PdWRKY genes showed higher expression levels in roots while in contrast results were observed in shoot tissues. Moreover, in date palm WRKYs candidates can be used for functional genomics, followed by potential use in a program of stress breeding.

Selection pressure and genetic diversification

Events in gene duplication like segmental, tandem and whole genome duplications play a key role in the complexities and expansion of gene families (Liu *et al.*, 2011). Tandem duplications have expanded WRKY family as reported by da Silva *et al.* (2017) and Zhang *et al.* (2017) in sweet orange and potato while segmental duplications are more common for the expansion of WRKY family than tandem duplications in tomato, soybean and cabbage (Cai *et al.*, 2013; Guo and Qiu, 2013). The purification selections in *A. thaliana* and *M. truncatula* have been reported by Wang *et al.* (2011); Ding *et al.* (2015). Interestingly, in date palm all the paralogous pairs of genes were observed to be went through by purifying selection.

CONCLUSION

To be concise, the current study identified 66 non-redundant WRKY proteins in date palm. These proteins were classified and characterized according to their conserved domains and gene structure subjected to comparative phylogenic analyses. These analyses indicated that the WRKY classes are conserved across different plant species. The majority of these genes were found to be expressed at higher levels in drought and salt environments. This suggests that they play a role in stress responses in date palm. In summary, this analysis provides valuable insights into the functional characteristics of WRKY TFs, particularly in the context of abiotic and biotic stress response. The identification and characterization of the candidate PdWRKY genes can be a valuable resource in stress breeding programs, as they may contribute to the development of more resilient date palm varieties.

AUTHOR CONTRIBUTIONS

Shahneeza Aimal Nadeem: Writing – original draft, Writing – review & editing, Formal analysis Mahad-ur-Rehman: Writing – original draft, Writing – review & editing, Formal analysis. Ikhlas Shafique: Conceptualization, Validation, Supervision, Resources, Validation. Hamna Chragh: Writing – review & editing. Ayesha Khalid: Writing – review & data analysis. Muhammad Bilal Mustafa: Writing – review & editing. Danish Raza: Validation, Writing – review & editing.

CONFLICT OF INTEREST

There is no potential conflict of interest in research and manuscript.

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