BIO-COMPUTATIONAL ANALYSIS OF WRKY TRANSCRIPTION FACTOR IN *VITIS VINIFERA*

Virendra Kumar[*] and A. K. Jaitly

Department of Plant Science, M.J.P. Rohilkhand University, Bareilly (U.P.) India.

Abstract

Stresses are major rate-limiting factors for *Vitis vinifera* growth and influence each stage during their life span. The signal transduction mechanism of plants during stress perception uses molecular components to make a stable decision "how to grow and develop by successful utilization of available resources". Transcription factors and miRNAs play a crucial role during stress responses in plants. The 57 unevenly distributed WRKY genes were identified in *Vitis vinifera*. The transcription factors further subjected to physiochemical properties, motifs, gene structure, phylogenetic and post-translational modification sites analysis. The protein motifs, gene structure, the phylogenetic analysis would be helpful to understand the relationship among the TF and also provided the evidence to subdivide them into groups based on the presence of motif and gene structure. The information also would be valuable in overcoming challenges in cultivating grapevine.

Key words: WRKY, Grapevine, Chromosome, PTMs, Stress, Molecular Components.

Introduction

The world population is increasing at an alarming rate and expected to reach about nine billion by the end of the year 2050 (Licausi *et al*., 2010). To feed the growing population needs more food to be grown, which is a challenging task. This can only be achieved by an increase in crop productivity per area as agricultural land is also decreasing due to urbanization. Plants cannot move from unfavorable place to favorable place (environmental conditions) so are exposed to a different type of stress when completing their life cycle (Evrard *et al*., 2013). "Stress can be understood as a stimulus or influence that is outside the normal range of homeostatic control of a given organism". The research of last decades showed that the global climatic pattern is becoming more unpredictable due to elevated CO_2, global warming, high salinity and chemical pollutions. Stresses are major limiting factors to agriculture practices worldwide (Qin *et al*., 2011). During the cores of evolution, the terrestrial plants develop some complex molecular mechanisms to overcome stresses (Levitt *et al*., 1980). Plants developed specific category protein-encoding genes that could regulate signal transduction and stress-responsive gene expression (Akhtar *et al*., 2012). The activation and

repression of TF target genes in a living cell regulate the global gene expression program (Liu *et al*., 2013).

WRKY transcription factor family is one of the largest families represented by 99 genes in *Oryza sativa* and genes in Arabidopsis and (Miao *et al*., 2004; Eulgem and Somssich, 2007). The WRKY transcription factors have a 60 amino acid residue long conserved DNA-binding domain (Motif). The WRKY transcription factor named based on the presence of highly conserved WRKYGQK amino acid sequence in motifs. The WRKYGQK motif binds to W-Box of genes to modulate their expression. Thus we can say that a single WRKY transcription factor can manipulate Abiotic or biotic stress-related pathways.

Grape (*Vitis vinifera*) is economic important woody perennial fruit crop species cultivated all over the world. Small genome size, Diploid inheritance, Facial clonal propagation, rapid growth, Small reproductive maturity period and phenotypically divergence make *Vitis vinifera* a model fruiting plant organism for functional genomics. The public release of the genome sequence provided an opportunity for genome-wide analysis in *Vitis vinifera* (Jaillon *et al*., 2007). The 57 putative WRKY genes were identified in the *Vitis vinifera* genome. The TFs subjected to the detailed analysis of their physiochemical properties, chromosomal distribution, motifs arrangement and

*Author for correspondence : E-mail: virenmtech@gmail.com

phylogenetic analysis. The WRKY genes also investigated for miRNAs interaction patterns and Post Translational modification sites analysis. The results would be useful for further studies of WRKY family protein regulation in vitace and the information also would be valuable in overcoming challenges in cultivating grapevine.

Materials and Methods

The experimental work was completed in the following segments

1. Identification, Distribution and Physicochemical properties of WRKY TFs Vitis vinifera:

The putative WRKY transcription factors were identified by three approaches. Initially, WRKY homologous peptides from *Vitis vinifera* were identified by balstp Search of pre-identified amino acid sequences encoding WRKY transcription factors against the PHYTOZOME database using default parameters. Moreover, the HMM profile of WRKY Protein family domain (PF03106) from Pfam was used as queries for the HMMER (v 3.1b1) (http://hmmer.org/) software against the whole genome sequences of the *Vitis vinifera* database (El-Gebali *et al*., 2019; Johnson *et al*., 2010). Similarity searches of *Vitis vinifera* genome and stand-alone EST sequences were performed using TBLASTN. (Altschul *et al*., 1990). The candidate sequences with E-values $\leq 1 \times 10^{-10}$ were retrieved and redundant sequences were removed using a stand-alone bash script. The non-redundant sequences were analysed by Pfam and SMART tools for the WRKY domain.

The BLASTP tool was used to determine the position of these WRKY protein-coding genes on *Vitis vinifera* chromosomes (Altschul *et al*., 1990). The WRKY family genes were mapped to the different chromosomes according to their physical distances in the GFF genome files. The candidate proteins were named using the species abbreviation of the source organism and the positions on the chromosomes and finally visualized using the Circos tool (Krzywinski *et al*., 2009).

The Physicochemical Properties of the above sequences, including the length of proteins, Aromaticity, isoelectric point (pI), grand average of hydropathy (GRAVY), molecular weight (Da) and instability index were determined using in-house python script.

2. Phylogenetic, Motif Composition and Genomic Analysis:

The WRKY proteins of *Vitis vinifera* were aligned using the ClustalW software with default settings. The alignment file was used to illustrate the evolutionary relationships of WRKYs in *Vitis vinifera*, a neighbor-

joining (NJ) phylogenetic tree was generated using a bootstrap test with 1000 replicates. Conserved motifs were identified using MEME (v4.12.0) with the following parameters: number of repetitions, any; the maximum number of motifs, 20; and optimum width of each motif between 6 and 300 residues. Each motif with an E-value $< 1 \times 10^{-10}$ was retained for motif detection (Bailey *et al*., 2009).

The EST and their corresponding genomic DNA were used in the Exonerate cdna2genome tool with the best one alignment (Slater and Birney, 2005). The result of cdna2genome was processed with the help of an in-house Perl script to obtain the Gff3 file. The Gff3 file was used to obtain the number of introns in each gene of the transcription factor. The WRKY gene and protein structures of *Vitis vinifera* were arranged corresponding to the phylogenetic tree in visualization.

3. Computational Identification of miRNAs Targeting the WRKY Gene:

To understand the function of miRNA in gene regulation pre-miRNA sequences of plants were used for the identification of targeting the WRKY genes. The web-based psRNATarget server was used to align all known plant miRNAs with the assembled transcripts of the WRKY gene with default parameters (Dai *et al*., 2018). The Cytoscape tool was used for graphical visualization of their interactions (Shanoon *et al*., 2003).

4. Post Translational Modification Sites Analysis in WRKY transcription factors of Vitis vinifera:

The ModPred tool was used to predict post-translational modification hotspots in stress regulation transcription factors family members protein sequence. The Modpred results were analyzed and processed for Heatmap generation.

Results and Discussion

1. Identification and Multiple Sequence Alignment of WRKY Family Genes:

The analysis shows that 60 possible protein-coding genes were found in *Vitis vinifera*. Out of 60, three genes were excluded from further analysis due to the lack of the conserved *WRKY* domain. The remaining genes were designated from Vvi*WRKY*01 to Vvi*WRKY57* according to their genomic location in *Vitis vinifera* chromosomes.

2. Genomic Distribution and Physicochemical properties:

The 57 *WRKY* genes unevenly distributed throughout the *Vitis vinifera* genome, However no *WRKY* genes detected on chromosomes 3 and the number of WRKY genes on the chromosome was not related to its length

(Fig. 1). Moreover, Two *WRKY* genes (Vvi_WRKY56 and Vvi_WRKY57) were located on undetermined chromosomes. The chromosome 4 is most abundant for WRKY TFs with the presence of 8 genes followed by chromosome 7 with six WRKY genes, chromosome 12 with Five genes, Chromosome 8, 10 and 15 each with four WRKY genes, Chromosome 1, 14 and 19 each with three WRKY genes, chromosome 2, 6, 11, 13 and 17 each with two WRKY genes and chromosome 5, 9, 16 and 18 each has one WRKY gene. Besides all of these, the *uncharacterized* portion of chromosome one also comprises one WRKY gene (Fig. 1). The VviWRKY

genes vary substantially in encoded proteins and their physicochemical properties. the average length of WRKY transcription factor family proteins was 373.44 amino acids and the Vvi WRKY13 has 136 amino acids therefore recognized as the smallest WRKY TF while Vvi WRKY55 holds the highest number of amino acids (746). The average aromaticity of bHLH TFs protein was 0.07.

However, Five Vvi WRKY TFs showed the lowest (0.04) aromaticity and the Vvi WRKY16 TFs showed 0.13 aromaticity, therefore, the WRKY TF element with lower aromaticity was not recognized. The average
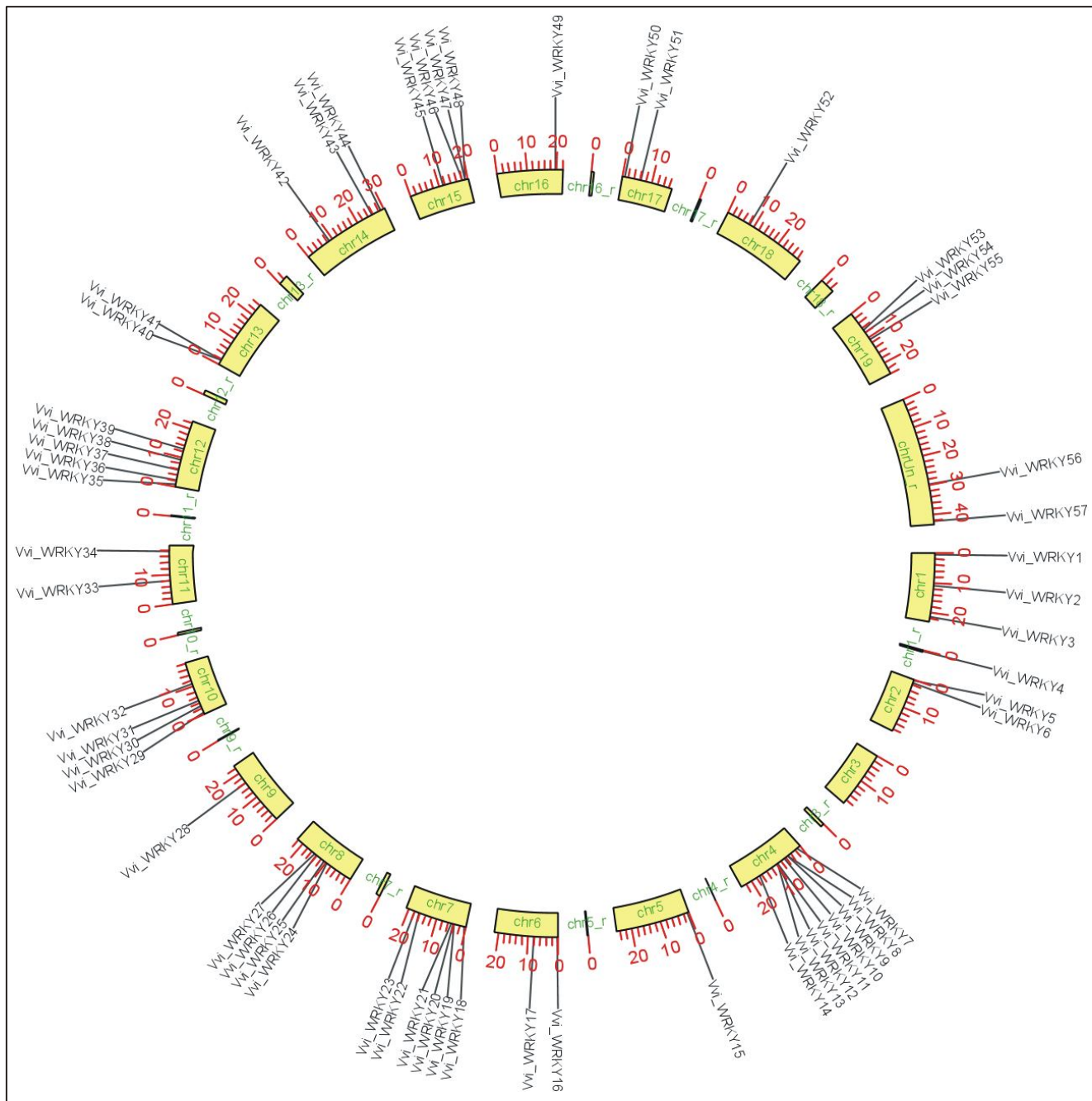

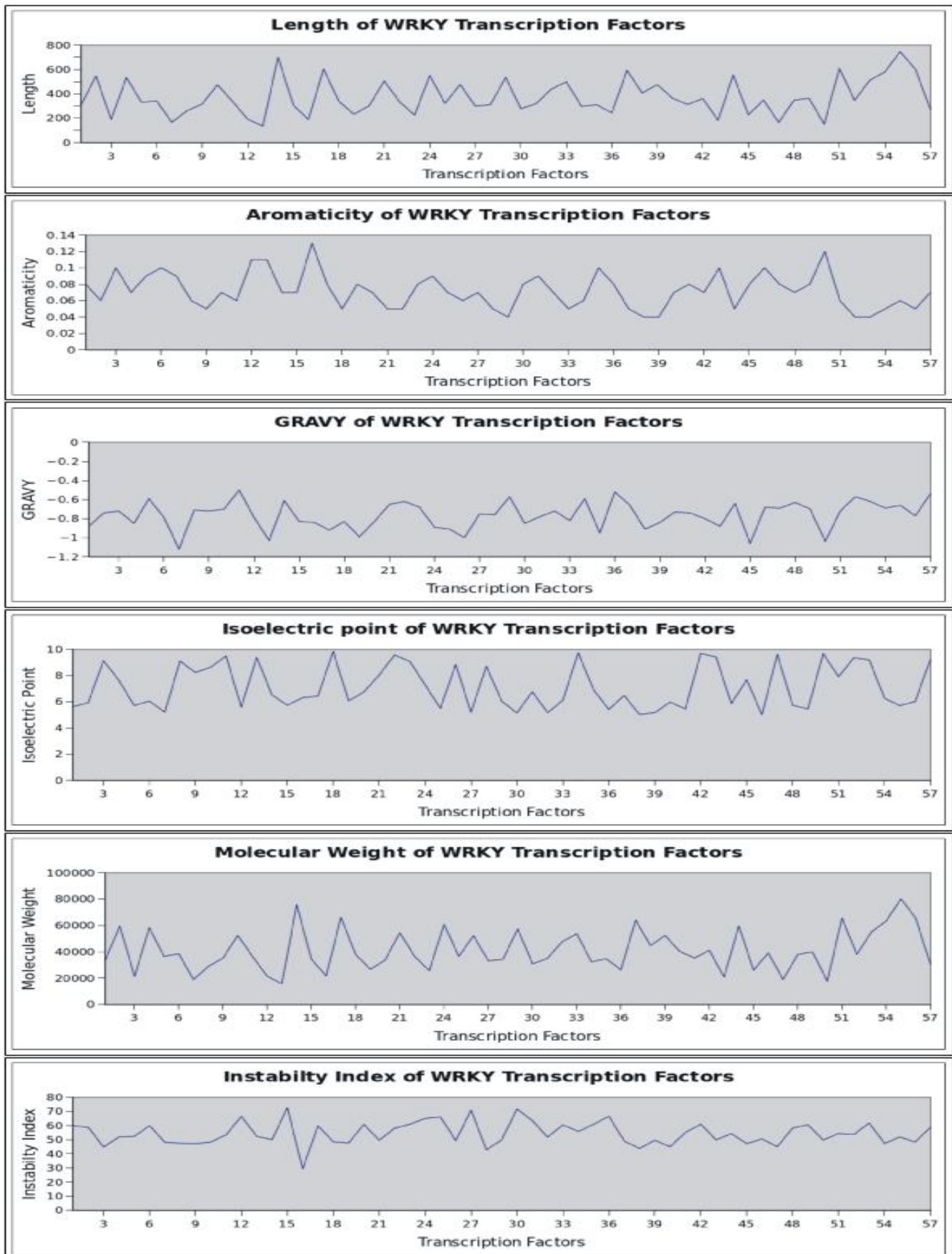**Fig. 1:** The distribution of WRKY transcription factors on different chromosomes of *Vitis vinifera*.

Fig. 2: Plots of Physio-chemical properties of WRKY transcription factors of *Vitis vinifera*.
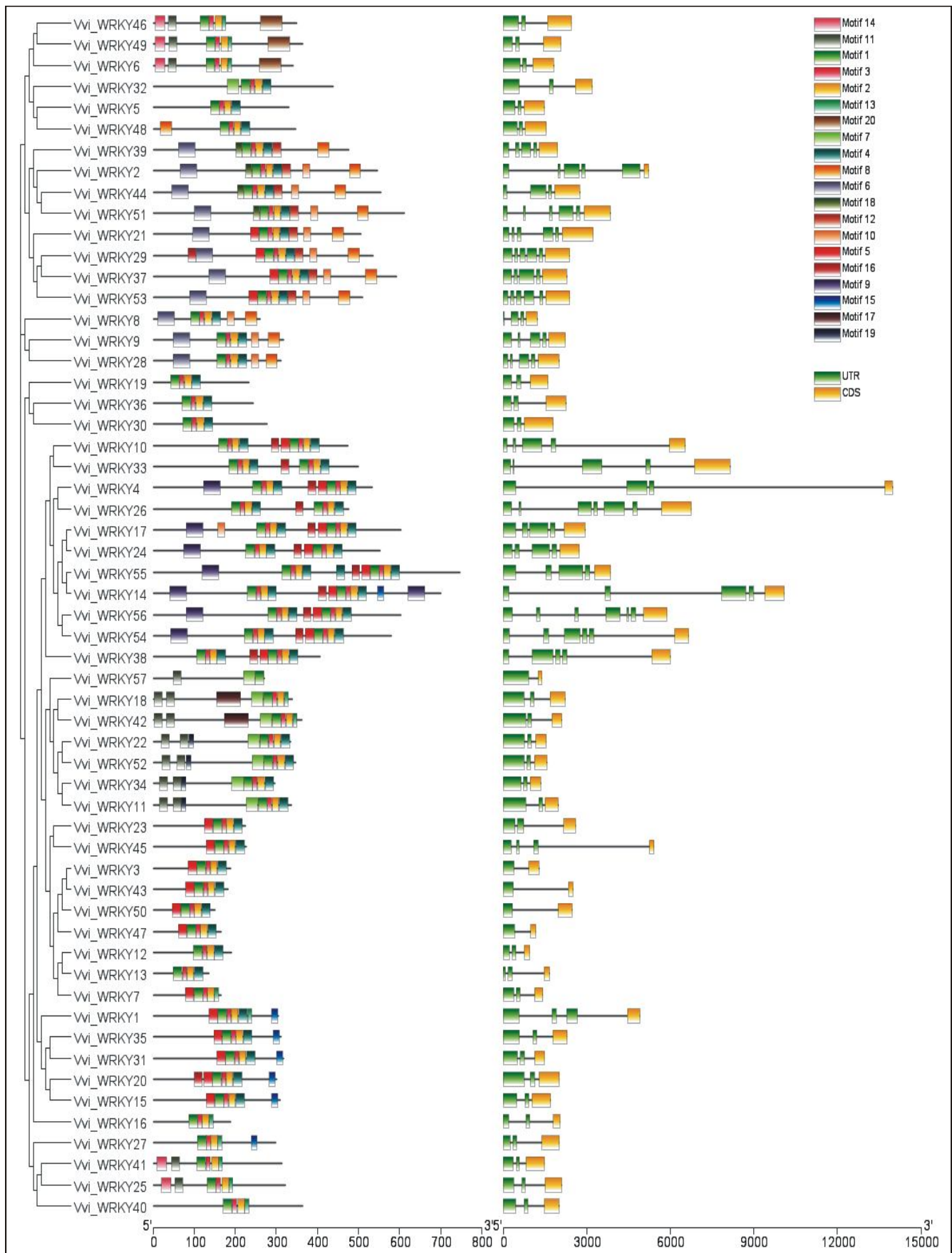
Fig 3: The gene and protein structures of WRKY TFs of *Vitis vinifera* corresponding to the phylogenetic tree.

GRAVY of 57 WRKY TFs protein was -0.77. However, with -1.12 GRAVY the Vvi WRKY7 stand as the minimum GRAVY protein of WRKY TF protein and Vvi WRKY11 with -0.5 GRAVY was the maximum GRAVY protein. The average isoelectric point of WRKY TFs protein of *Vitis vinifera* was 7.13 pH. However, with pH 5.01, Vvi WRKY46 TF stands as the lowest one isoelectric point protein and with 9.84 pH, TF protein Vvi WRKY18 stands as the highest one.

The molecular weight of 75 WRKY TF proteins ranged between 15758 to 80265.9 Dalton and the average molecular weight was 41126.14 Dalton. The Vvi WRKY13 TF was the lowest molecular weight protein with 15758 daltons and Vvi WRKY55 was the higher one with 80265.93 Dalton molecular weight. The average instability index of WRKY TFs protein was 54.31. The Vvi WRKY16 with a 29.36 instability index was the most stable protein in a test tube while Vvi WRKY15 TF protein with 72.5 instability index was the most unstable protein of the AP2/EREBP transcription factor protein family (Fig. 2). The WRKY TFs of *Vitis vinifera* peptide sequences, cDNA sequences and the values of physiochemical properties in the tabulated form given in an online supplementary file (http://dx.doi.org/10.17632/rfsxsrchts.1).

3. Phylogenetic, Motif Composition and Genes structure of WRKY TFs:

All putative (57) WRKY transcription factors amino acid sequences were subjected to multiple sequence alignment by ClustalW and the phylogenetic tree was constructed using the neighbor-joining method based on the p-distance model with 1000 bootstrap replicates. An unweighted, unrooted phylogenetic tree of 57 leaf with 112 unique splits including 54 unique non-trivial splits was generated.

The WRKY TF sequences were also analyzed for 20 conserved domains by MEME and the results further analyzed and summarized in tabulated form for motif name, Length, Occurrence, E-value and amino acid sequence of the motif (Fig. 4). The analysis revealed that the 22 amino acid long motif 1 was distributed 67 times followed by 67 time distribution of 17 amino acid long motif 2, 67 times distribution of 11 amino acid long motif 3, 57 times distribution of 21 amino acid long motif 4, 24 times distribution of 21 amino acid long motif 5, 18 times distribution of 20 amino acid long motif 11, 12 times distribution of 19 amino acid long motif 16, 12 times distribution of 10 amino acid long motif 13, 11 times distribution of 41 amino acid long motif 6, 11 times distribution of 28 amino acid long motif 8, 10 times distribution of 18 amino acid long motif 10, 7 times distribution of 41 amino acid long motif 9, 7 times distribution of 29 amino acid long motif 7, 7 times distribution of 21 amino acid long motif 12, 7 times distribution of 15 amino acid long motif 15, 5 times distribution of 24 amino acid long motif 14, 4 times distribution of 15 amino acid long motif 18, 4 times distribution of 12 amino acid long motif 19, 3 times distribution of 53 amino acid long motif 20 and 2 times distribution of 59 amino acid long motif 17 (Fig. 3). The motifs were highlighted with different colored boxes with numbers 1 to 20. The organization of WRKY transcripts has been shown to be highly variable in *Vitis vinifera*.

Motif	Length	Occurrence	E-value	Amino Acid sequence
1	22	67	5.1e-933	DILDDGYRWRKYGQKx[VI]K[GN][SN]PY
2	17	67	1.1e-471	CP[VA][KR]K[QH]V[EQ]R[SA]S[ED]DPS[IM][LV]
3	11	67	4.0e-397	PRSYY[RK]C[TS][SH]AG
4	21	57	8.7e-353	[TI]TYEGxHNH[PD]xPxARRASASS
5	21	24	4.9e-118	KKK[AG]E[KR][KTR][IV]R[EK]PR[VF][AV][FV]QT[RK]SEV
6	41	11	3.9e-097	Lx[SV][LA][KQ][AE]E[LM][EG][RE][MV][RN][EA]EN[EK][KR]LRxMLEQ[IMV]T[KN][ND]YNALQM[HQ][LFV]VE[LI]MQ
7	29	7	2.1e-080	SSGRCHCSK[RK]RKLRVKR[STV][IV][RK]VPAIS[NS]K[ILM]A
8	28	11	8.7e-071	[TL][VI][EA][AT]AT[AK][AS][IL]T[AS]DP[NS]FTAALAAAI[TS]SII[GL]
9	41	7	1.4e-051	[LP]PI[SA][RP]S[PS][YC][LF]TIP[PS]GLSP[TS]SLL[DE]SPV[LF]LS[NT][SM][KN][VA][ELQ]PSPTTG[ST]F
10	18	10	9.0e-033	[ASC][STP][IAL]SA[SV][AS][PS]FPT[IV]TLDLT[QHT]
11	20	18	4.2e-032	C[EQ]E[AL][ATV]D[AK][AIG][VL]S[SK]F[EK][KR][VLA][LI]S[LI]L[NS]
12	21	7	1.7e-024	TSAAASMLLSGS[MS][PST]S[QA][DP]GL[MG][NS]
13	10	12	1.4e-025	[EI][VIT]TY[ER]GRH[TN][CH]
14	24	5	6.8e-018	[WGT][DEQ][QRH][KN][RTN][LAV][IV][NDG]EL[TLV][QHNR]G[RM][EDS][LFM][AT][KN]QL[KQR][INS][HLV]L
15	15	7	2.2e-016	[DP][EHY]GLL[QE]D[ILM]VPS[FM][IM]R[KN]
16	19	12	1.2e-015	S[KR][GA]D[ED][DE][DE]ED[ED]P[ED]SKRRKKE
17	59	2	7.8e-015	QQMK[HY]QAD[MT]MY[RS]RSNSGINL[KN]FD[GS]SSCTPTMSSTRSFISSLS[MV]DGSVANLDGN[AS]FHLIG
18	15	4	1.4e-010	[HP][ALNV][KR][KR][AC]RVSVRARC[DQ]T
19	12	4	1.9e-010	RTGHARFRR[AG]P[LV]
20	53	3	2.9e-010	[IMN][DEY][ND][NS][LI][LMV]G[CGN][FY][SP][PT][ST]F[ILV]SPA[TA]S[DER]SN[YC]F[SP][LMV]S[PS][CD][QE]M[NS][SN][FL]G[GM][GNP][HPQ][NS][LV][QY][AHT][PST]E[SA][DEN]L[TN]E[IN]ISA

Fig. 4: Length, Occurrence, E-value, and Amino Acid sequence of 20 conserved motifs of WRKY TF of *Vitis vinifera*.
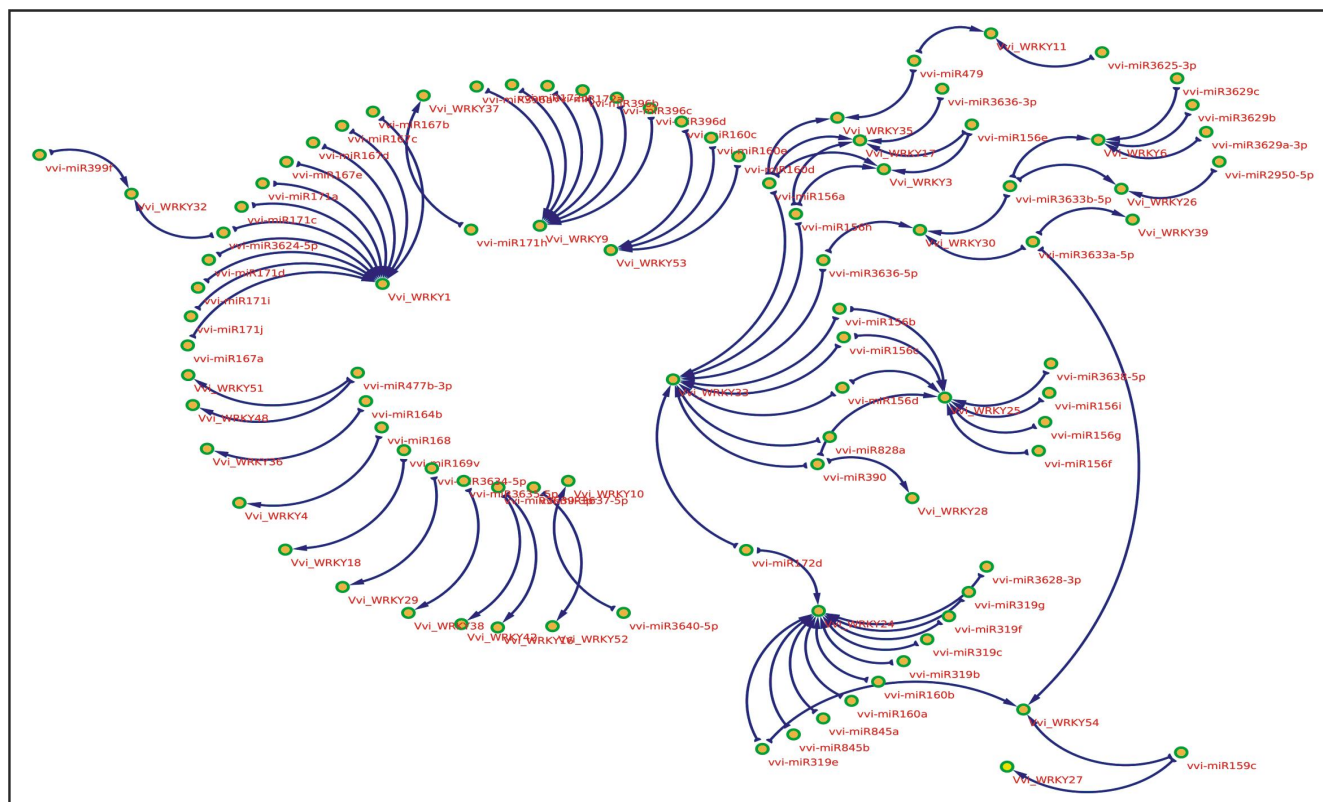
Fig. 5: Interactions map of WRKY transcription factors and miRNAs of *Vitis vinifera*.

The Vvi WRKY13 encoded the smallest bHLH TFs of *Vitis vinifera* containing only 411 nucleotides (136 amino acids). Similarly, the Vvi WRKY26 contained the largest transcript, encoding an open reading frame (ORF) of 2945 nucleotides (477 amino acids). However, the largest NAC TFs protein was Vvi WRKY55 with 746 amino acids. The average length of introns is 589.58 base pairs long and their organization in TFs was very dynamic, ranging from one to six introns per gene. The number of *Vitis vinifera* WRKY transcription factors that contained various numbers of introns as follows: one (5), two 28), three (5), four (11), five (6) and six (2) fig. 3.

4. Interactions of TFs with miRNAs:

The psRNA Target Analysis Server used to analyse the interaction between putative WRKY genes and miRNAs given in fig. 5, which showed that 32 WRKY genes may be regulated by 68 miRNAs. The regulation of TFs was very dynamic, ranging from one to four genes per miRNAs. The number of *Vitis vinifera* miRNAs which controls the regulation of WRKY TF was as follows: fifty one miRNAs control the regulation of one TFs, ten miRNAs (e.i. vvi-miR156b, vvi-miR156c, vvi-miR156d, vvi-miR156e, vvi-miR172d, vvi-miR319e, vvi-miR3624-5p, vvi-miR3636-5p, vvi-miR477b-3p and vvi-miR479) control the regulation of two TFs, six miRNAs (e.i. vvi-miR156h, vvi-miR159c, vvi-miR3633a-5p, vvi-

miR3633b-5p, vvi-miR3639-3p and vvi-miR390) control regulation of three TFs, one miRNAs (e.i. vvi-miR156a) control the regulation of four TFs (Fig. 5).

5. Post Translational Modification Sites analysis in TF proteins:

PTMs interfere with protein regulation and function. However, their characterization for structural and functional signatures has been hindered by the scarcity of data. Therefore the WRKY TFs subjected to the ModPred tool (http://www.modpred.org/) to predict twenty-one PTMs sites. The analysis revealed a total of 16680 putative PTMs hotspots in 57 peptide sequences, out of which 7859 PTMs belonged to Proteolytic cleavage followed by 1730 for Phosphorylation, 1083 for O-linked glycosylation, 920 for Amidation, 853 for ADP-ribosylation, 660 for Methylation, 619 for Ubiquitination, 579 for Acetylation, 576 for Hydroxylation, 493 for Carboxylation, 301 for SUMOylation, 282 for N-linked glycosylation, 243 for Disulfide linkage, 40 for PUPylation, 83 for Pyrrolidone carboxylic acid, 81 for Palmitoylation, 67 for GPI anchor amidation, 65 for Sulfation, 39 for N-terminal acetylation and 7 for C-linked glycosylation.

The PTMs analysis generated a lot of data practically it has not been possible to present all the data on paper, therefore a heatmap was generated using the name of PTM along with each TF (Fig. 6).
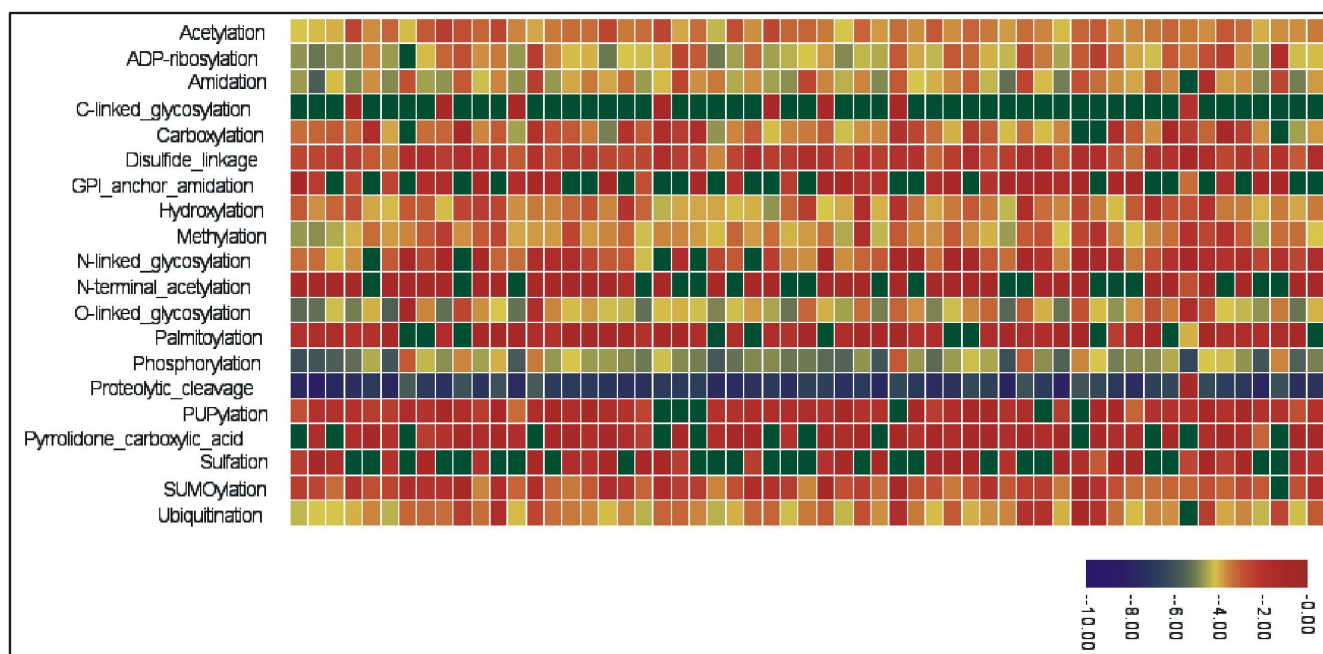
Fig. 6: Heatmap showing the distribution of Post-translational modification sites of WRKY transcription factors of *Vitis vinifera.*

During the changing environment, the protein's PTMs is a key process that integrates plant growth and helps to diversify the limited genome of organisms. PTMs govern transcription factors genes regulation by the activation or deactivation of stress sensors through PTMs, which ultimately leads to the expression of hundreds of genes.

Conclusion

In summary, a total of 57 WRKY TF family genes were found to be present in *Vitis vinifera* and their distribution on chromosomes independent from its length. In order to characterize the physicochemical properties of WRKY TF, six parameters have been selected: peptide length, Aromaticity, GRAVY, isoelectric point (pI), Molecular weight and Instability Index. The physiochemical attributes of the amino acids are important for protein fold recognition and also strongly associated with their roles in a cell (Sharma *et al*., 2013). Theoretical pI and Mw of peptide sequence help to determine the approximate area of a 2D-gel where a protein of interest may be detected. The GRAVY simply indicated the polarity of the protein and the instability index calculation estimated the stability of the protein in the test tube (Wang *et al*., 2019). Exploring the structural and functional properties of TF was essential in concern to develop a robust understanding of their function. The phylogenetic analysis used to elucidate how TF genes evolve and come to the way they are today, It is also useful to predict how they will change in the future. Phylogenetic, Motif composition and Genomics helps to understand the relationship among the TF and also provided the shreds

of evidence to subdivide them into groups based on the presence of motif and gene structure. The understanding of these TF has been an asset and boon to many agricultural and horticultural activities globally. To create new protein capacities, a novel domain structure emerges through gene duplication, recombination, fission, fusion and rearrangement events. Like other proteins TFs also comprised several conserved portions.

A total of 21 types of PTM sites were detected in the WRKY TF Family of *Vitis vinifera.* The PTMs are the fastest, earliest and versatile regulatory process of plant responses to changes in the environment and have been implicated in the regulation of several regulatory and metabolic processes (Borjana *et al*., 2018). They are also responsible for Plant immune to achieve a response that is appropriate to the type of environmental pressure (Withers and Dong, 2017). The PTMs in crop plants under field conditions maintained robustly homeostasis under extreme conditions and helped to diversify the limited genome of organisms (Green and Garneau-Tsodikova, 2010). They also helped plants to make a stable decision to grow and develop by the successful utilization of available resources. The advances in PTM study need to be examined more closely to biotic or abiotic stresses in plants. The effect of PTM on the activity and assembly of transcriptional complexes and subcellular locations of substrates are important areas to ensure adequate agricultural production in the future (Hashiguchi and Komatsu, 2016).

The availability of large volumes of genome-scale data and advanced computational techniques enabled to

dissect the complex nature of the stress response. The plethora of novel insights reported the overarching roles of major stress regulatory TFs, miRNAs and PTMs, which are central to the fine-tuning of stress response pathways. The study contributes to understanding the gene expression programs and transcriptional regulation in this economically important crop plant. Additionally, The genetic determinant identified in this study is likely to be involved in cross-talk between biotic and abiotic stress responses.

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