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In-silico analysis of WRKY Transcription Factors and WRKY associated genes in rice wild species *Oryza coarctata* and *Oryza nivara*

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Abstract

Transcription factors (TFs) are pivotal in regulating gene expression, with the WRKY superfamily being prominent in plants. We explore the evolutionary landscape and functional diversity of WRKY TFs in two wild rice species, Oryza coarctata and Oryza nivara, compared to cultivated rice (Oryza sativa). Utilizing genomic data and computational methods, we identify WRKY TFs and analyze their phylogenetic relationships, revealing evolutionary patterns and similarities between the wild species and O. sativa. The WRKY TF family demonstrates extensive distribution across chromosomes, with specific clustering observed. Notably, O. coarctata and O. nivara exhibit distinct WRKY TF profiles despite their relatedness. Moreover, we uncover the regulatory networks associated with WRKY TFs, elucidating key genes and interactions involved in plant growth, development, and stress responses. Through network analysis, WRKY TFs emerge as central regulators, with WRKY71 notably regulating various stress responses in rice. Additionally, WRKY TFs like WRKY53, WRKY62, and WRKY28 are implicated in salinity stress and defense mechanisms, underscoring their multifaceted roles in plant adaptation. Furthermore, we investigate the structural features of hub genes, shedding light on their functional significance. Comparative protein structure analysis reveals conserved domains and structural similarities across wild and cultivated rice species, indicating evolutionary conservation in most of the genes, while in a few significant structural variations were observed. These findings offer valuable resources for further exploration of WRKY-mediated regulatory networks and genetic engineering strategies aimed at enhancing crop resilience and productivity in changing environments.

Keywords: Rice; Oryza coarctata; Oryza nivara; WRKY; Stress response

Introduction

In living organisms, transcription factors (TFs) play an essential role as regulatory mechanisms in controlling the transcription of downstream target genes. Many groups of transcription factors (TFs) in plants are in charge of the general regulatory systems that govern development and stress (Mahiwal et al., 2024). The WRKY TF superfamily, which is made up of many WRKY TF members, is the second biggest of these in plants. Thus far, the genome-wide study has found 74 WRKY genes in the model plant *Arabidopsis thaliana*, more than 100 genes in *Oryza sativa*, 197 genes in *Glycine max*, and 81 WRKY members in *Solanum lycopersicum* (Ross et al., 2007; Rushton et al., 2010; Huang et al., 2012; Jiang et al., 2017). The majority of protein families are identified by a single, distinct functional domain that is shared by all members of the family. Because of the "WRKY" domain's high degree of conservation, the WRKY superfamily was designated (WD) (Goyal et al., 2023). This sixty-amino acid domain has a unique zinc-finger-like motif at the C-terminus and a conserved amino acid sequence motif at the N-terminus called WRKYGQK (Cheng et al., 2019). In addition to WD, the basic nuclear localization domain, leucine zippers, glutamine-rich region, proline-rich region, serine-threonine-rich region, kinase domain, and TIR-NBS-LRR domain are found in WRKY proteins (L.Chen et al., 2012). On the other hand, changes to the heptapeptide WRKY sequence impact the interaction network of the WRKY protein by changing its three-dimensional structure. A further noteworthy mutation-based investigation on OsWRKY7 with a WRKYGKK signature motif (rather than WRKYGQK) revealed an increased TF binding affinity towards the W-box upon changing "GKK" to "GQK" (X. Chen et al. 2019).

Compared to its important role found in the field of biotic stress responses, the significance of WRKY in abiotic stress was understood much later. Thus far, indirect transcriptional profiling has made it possible to determine a hint regarding WRKY's possible function in abiotic stress tolerance (Strader et al., 2022). When OsWRKY11 was overexpressed in rice under the HSP101 promoter, it was shown to have improved drought tolerance, with slower leaf withering and a higher survival rate of green plant portions (Wu et al., 2009). Comparable outcomes were observed with modified drought and salt tolerance levels when OsWRKY45 and OsWRKY72 were overexpressed in Arabidopsis using a constitutive promoter CaMV35S. The activation of ABA/stress-related genes may be the cause of this enhanced resistance to salt and drought (QIU & YU, 2009; Song et al., 2010). OsWRKY50, a transcriptional repressor, regulates ABA-independent, salt stress tolerance as well as ABA-dependent seed germination and seedling growth (Huang et al., 2021).

Using both its overexpressing and mutant lines, OsWRKY21's critical role in controlling internode elongation and plant height in rice was shown. In comparison to the wild type, OsWRKY21 overexpression lines exhibit a semi-dwarf phenotype, an early heading date, and short internodes; in contrast, the CRISPR/Cas9 mutant exhibited the exact opposite phenotype (Wei et al., 2021). While OsWRKY29 overexpression resulted in the opposite *viz.* decreased seed dormancy and ABA hyposensitivity, OsWRKY29 knockout and RNA interference lines enhanced seed dormancy and were hypersensitive to ABA. OsABF1 and rice VIVIPAROUA1 (OsVP1), two ABA-positive sensitive genes, were increased in OsWRKY29 mutants but downregulated in OsWRKY29 overexpression lines. OsWRKY29 attaches itself directly to W-boxes in OsABF1 and OsVP1 promoters. OsWRKY29, thus, plays a crucial role in inhibiting seed dormancy by lowering ABA responsiveness (Zhou et al., 2020). It was determined that OsWRKY36 and OsWRKY102 decrease cell wall lignification in rice based on notable alterations in the culm shape of the double mutants of OsWRKY36/OsWRKY102 compared to corresponding single-mutant lines and wild-type control (Miyamoto et al., 2020). The present study is aimed at understanding the evolution of WRKY TFs and their associated genes *in-silico* in two wild rice species *viz. Oryza coarctata* and *Oryza nivara* from the cultivated species.

Materials and Methods

Data acquisition for analysis

The list entire TFs of rice were downloaded from Plant Transcription Factor Database (PlantTFDB), developed by (Jin et al., 2017). WRKY family IDs were used to retrieve the protein sequences and other information from the Rice Annotation Project database (RAPDB) (Sakai et al., 2013). The entire protein sequences of the two wild species, *Oryza coarctata* and *Oryza nivara* were downloaded from National Genomics Data Center (NGDC), Beijing Institute of Genomics, Chinese Academy of Sciences and Ensembl plants database respectively (Yates et al., 2022; Zhao et al., 2023). The chromosomal location of these WRKY TFs was used to visualize the distribution of these genes using the map tool available in Oryzabase (Kurata and Yamazaki, 2006).

WRKY TFs retrieval from wild rice species

BLASTP using Diamond Aligner (Buchfink et al., 2015) was run for the WRKY Tf proteins obtained from RAPDB against *O. coarctata* and *O. nivara* with an E-value threshold of 1e-5 and query coverage of >80%. Domain prediction of the protein sequences from *O.*

coarctata and *O. nivara* was done using the standalone version of Interproscan (Jones et al., 2014) and those with the WRKY domain were used for further characterization.

Phylogenetic Analysis

The WRKY domains and the full-length protein sequences of the two wild species were used to analyze the phylogenetic relationship of the gene family separately. The amino acid sequences were aligned using standalone version of MUSCLE with default parameters. The unrooted neighbour-joining (NJ) phylogenetic tree was constructed using MEGA v11 (Tamura et al., 2021) with 1000 bootstrap replications.

Unweighted gene network analysis

Individual gene networks of the WRKY TFs were retrieved from the STRING database (Szklarczyk et al., 2023). A confidence score of 0.7 was set to retrieve the network containing only those genes with stronger interaction. The resulting interaction file of the STRING database was utilized to narrow down some of the key WRKY TFs and genes associated, with Cytoscape (Shannon et al. 2003). The interaction files obtained for each WRKY TF were loaded into Cytoscape and merged into a single network based on the presence of overlapping genes. Preliminary network analysis was performed to check the optimal network properties, such as clustering coefficient and node degree distribution. Further, we used a plugin CentiScaPe (Scardoni et al. 2014), that allows topological analysis of the directed/undirected/weighted networks for identification of topmost nodes (genes), by calculating centrality indices. For our study, we chose degree centrality to identify the hub genes.

Protein tertiary structures analysis of hub genes

Amino acid sequences of the hub genes with the cut-off degree centrality >= 10 were downloaded from RAPDB and BLASTp was performed against the protein sequences of the two wild rice species. All the protein sequences checked for signal peptides using InterProScan and their corresponding sequences were removed prior to structure prediction. The structure prediction was carried out using Robetta online structure prediction server developed by the Baker lab at the University of Washington. Robetta Comparative Modeling (Robetta CM) method was used for the same (Song et al., 2013). The predicted structures were downloaded in pdb format and their quality parameters were checked. To determine the structural similarity, the protein structures of *O. nivara* and *O. coarctata* were superimposed

using ChimeraX software (Pettersen et al., 2021), against their corresponding protein structures in *O. sativa* (Meng et al., 2020).

Results and Discussion

WRKY TFs play a diverse role in plant regulation ranging from growth and development to stress response (Jimmy & Babu, 2015). In rice, these WRKY TF family contains more than 100 genes (Gao et al., 2006) and are distributed across all the chromosomes, with the highest number of genes (25) present in chromosome 1 followed by chromosomes 5 and 4 which had 16 and 13 TFs respectively (Figure 1). This TF family is the second largest in the plant system (Song et al., 2023). A total of 108 WRKY TFs were obtained from the RAPDB database and their corresponding protein sequences (including splice variants) were also downloaded.

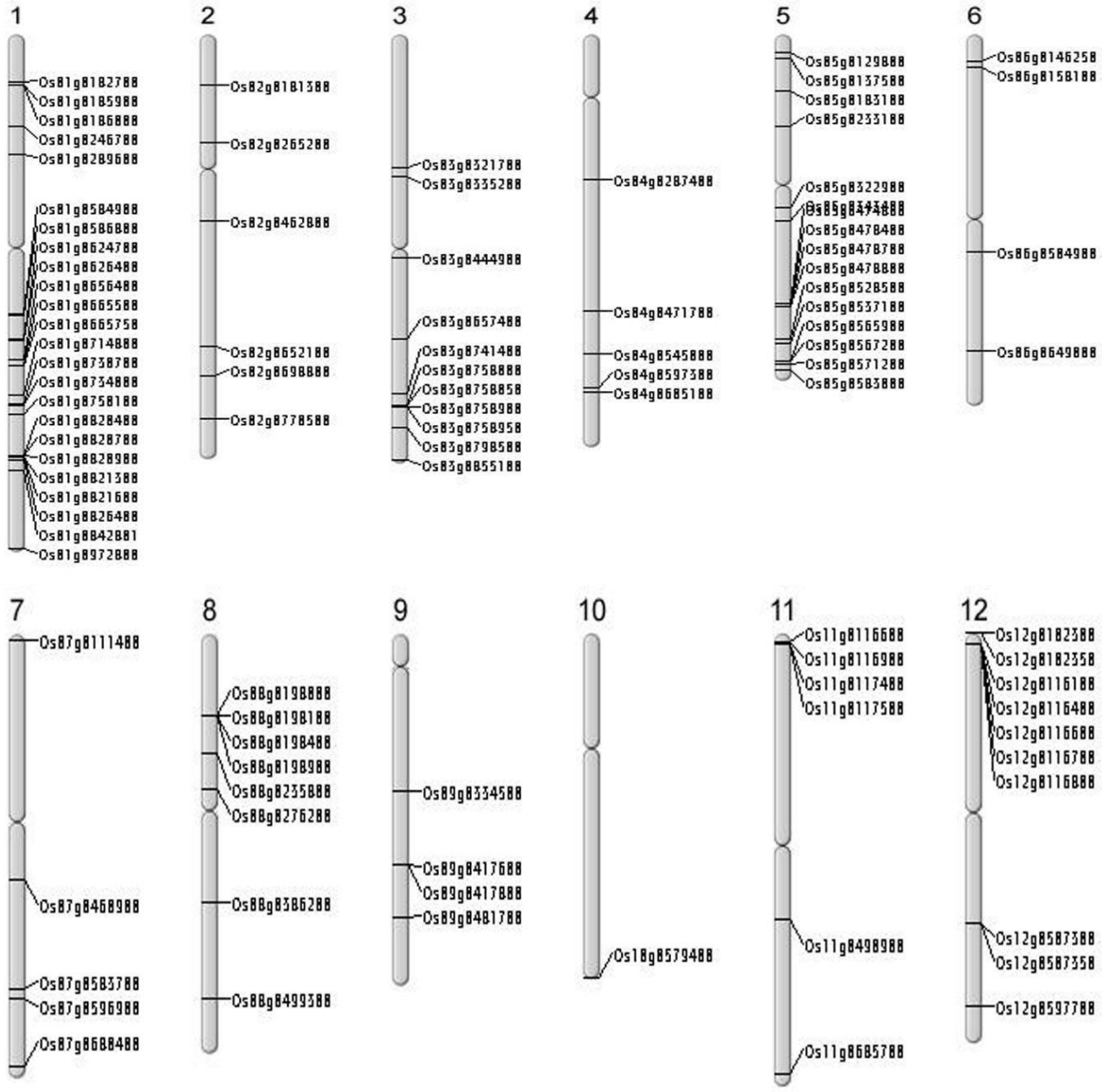

Fig 1. Chromosomal location of WRKY transcription factors of *O.sativa*

Diamond aligner BLASTp program was run for the downloaded *O. sativa* protein sequences against *O. coarctata* and *O. nivara*. A total of 5 hits per query (*O. sativa*) was given as the cut-off based on homology. Interproscan domain analysis revealed that *O. nivara* had 97 WRKY TFs whereas *O. coarctata* had 113 WRKY TFs. *O. nivara* has AA genome with 12 chromosomes, whereas *O. coarctata* has KK LL genome allotetraploidy (Xu et al., 2016; Zhao et al., 2023). Phylogenetic analysis of the WRKY TFs of *O. coarctata* and *O. nivara* was individually conducted using the MEGA11 software, and this analysis highlighted that both the species had two main subgroups with the smaller subgroup containing 4 and 3 WRKY proteins respectively, and the larger subgroup had further subdivisions (Figure 2A and 2B). The phylogenetic tree constructed by combining the WRKY proteins of both species showed that one of the 4 WRKY proteins falling under the smaller subgroup, GWHPCBHR008526 had similarity with ONIVA01G40310.1 present in the larger subgroup which corresponds to WRKY22 of *O. sativa* (Figure 2C). Moreover, similarity between different WRKY TFs were observed between the two wild rice species. Evolution of WRKY TFs and diversity in plants has its ancestry from algae, however, very few were found in algae as compared to plants (Rinerson et al., 2015).

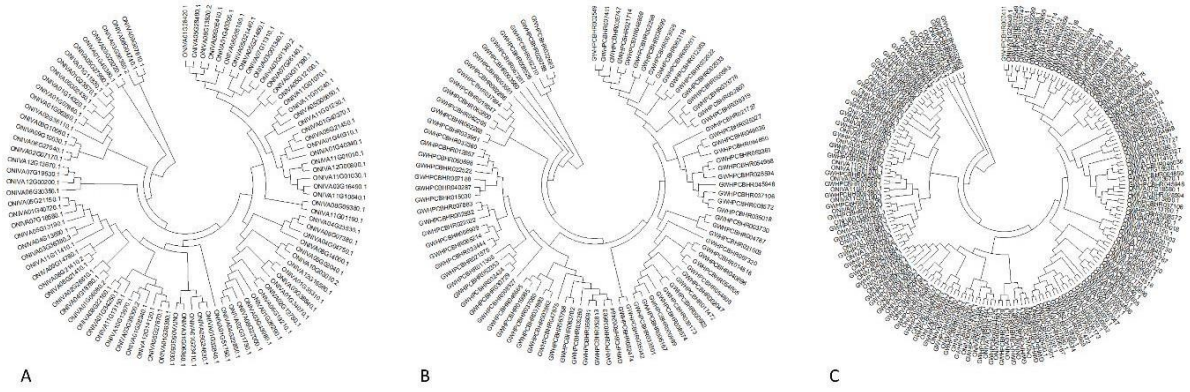

Fig 2. Phylogenetic analysis of WRKY TFs in (a) *O.nivara* (b) *O.coarctata* (c) between *O.sativa* and *O.coarctata*

The WRKY TFs not only play direct roles in plants' growth and development but also help in eliciting the expression of stress-responsive genes and mechanisms (Ulker & Somssich, 2004). In order to trace down the key WRKY TFs and genes associated with them, we constructed an unweighted gene network for each WRKY of *O. sativa* and merged them into one (Figure 3A). The merged network had 1447 nodes (genes) and 933 edges (interactions). Initial analysis of the merged network showed that the node-degree distribution followed the power law of distribution with R^2 value of 0.794 and the betweenness to degree correlation was significant (Figure 3B and 3C). To identify the hub genes, degree centrality parameter of the centiscape plugin was used. The degree centrality ranged from 16 to 1 and a cut-off of 10 was used for identifying the key regulators. The highest degree (16) was

observed for WRKY71 TF. In rice WRKY71 has been extensively studied for its role against biotic as well as abiotic stress tolerance (cold stress, brown planthopper infestation, bacterial blight, etc) (Kim et al., 2016; Li et al., 2024). Other WRKY TFs with higher degree centrality include *WRKY53*, *WRKY62*, *WRKY28*, *WRKY69*, *WRKY24*, and *WRKY7* with a degree of 15, 14, 12, 12, 12, and 10 respectively. These TFs are crucial players in salinity stress, defense response, bacterial blight, etc (Chujo et al., 2013; Xu et al., 2022). Other than WRKY,

different other transcription factors and genes were also found to have a higher degree of centrality. Transcription factors such as AP2/ERF (*ERF91*), zinc finger protein (*ZFP36*), GRAS transcription factor (*GRAS56*), and MADS box transcription factor (*MADS14*) had a degree of 16, 14, and 11 respectively. In addition, genes such as *PUB67*, *ATL69*, *PAL3*, *Fbox559* were also identified as significant hub genes. These TFs and genes are well known for their role in biotic and abiotic stress response and it could be inferred that these genes are associated with the WRKY TFs.

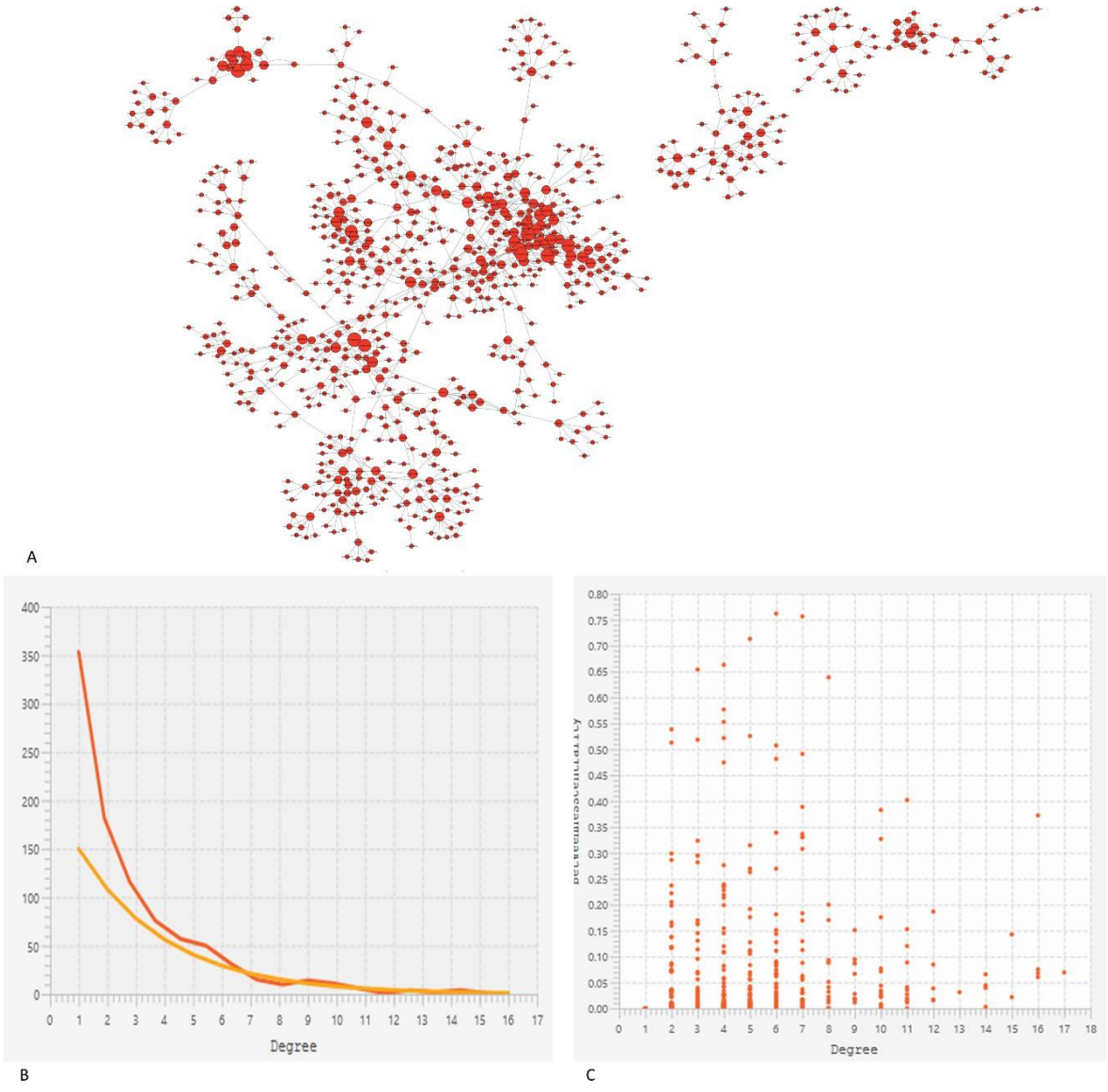

Fig 3. Network analysis of WRKY transcription factors present in *O.sativa* (a) merged

network of individual WRKY transcription factors (b) node-degree distribution of the merged network (c) betweenness to degree correlation of the merged network

To understand the structural variation of these hub genes, their protein sequences were retrieved from *O. coarctata* and *O. nivara.* Superimposing of the corresponding hub proteins against the two wild species showed structural similarity, however, a few proteins showed slight variations (Figure 4 and 5). For instance, in *WAK14, a* Wall-associated kinase protein which is involved in rice blast resistance (Delteil et al., 2016) *O. sativa* had 545 amino acids while *O. coarctata* had only 492 amino acid residues. Interestingly, *O. nivara* had triple the length of amino acid residues (1517). Due to this reason, the structure prediction software couldn't predict its structure. Notably, these variations are significant since there could be some underlying evolutionary benefits. Further characterization could be carried out to study the gain or loss of function of blast resistance due to *WAK14* of *O. nivara*. A few hypothetical genes were also identified as hub genes and therefore included in the structure prediction. One such hypothetical gene, Os01g0952900, showed close similarity in structure with *O. nivara* and not with *O. coarctata*. Even though the hypothetical genes haven't been functionally characterized in cultivated rice itself, the O. coarctata paralog could be an interesting candidate, since it is associated with WRKY TF. A few potential candidate genes were identified through our in-silico analysis which could be involved in stress responses from the two wild rice species. These could be functionally characterized and utilized in breeding programs and biotechnological tools.

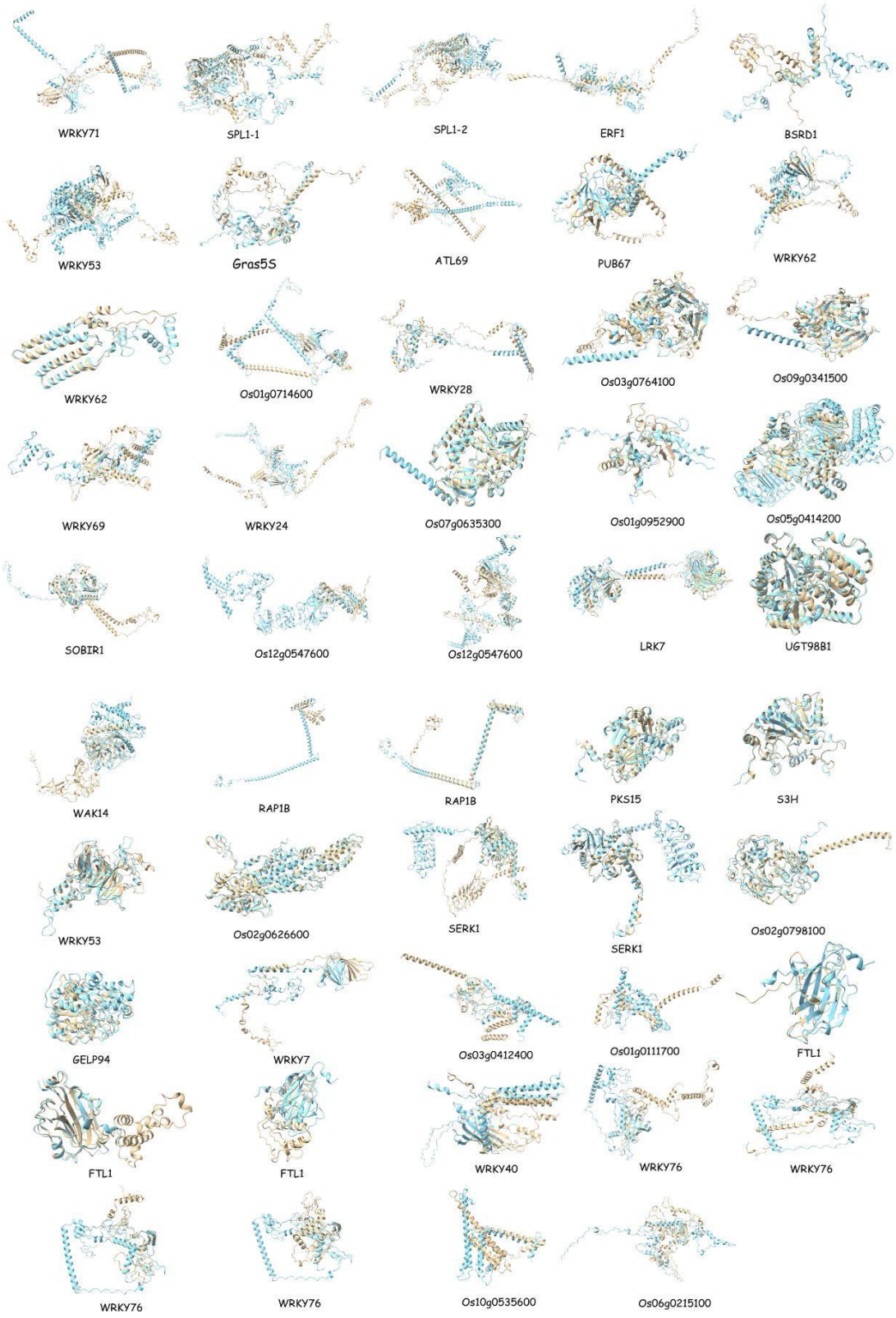

Fig 4. Protein structure prediction and superimposing for hub genes of *O.sativa* against *O.coarctata*

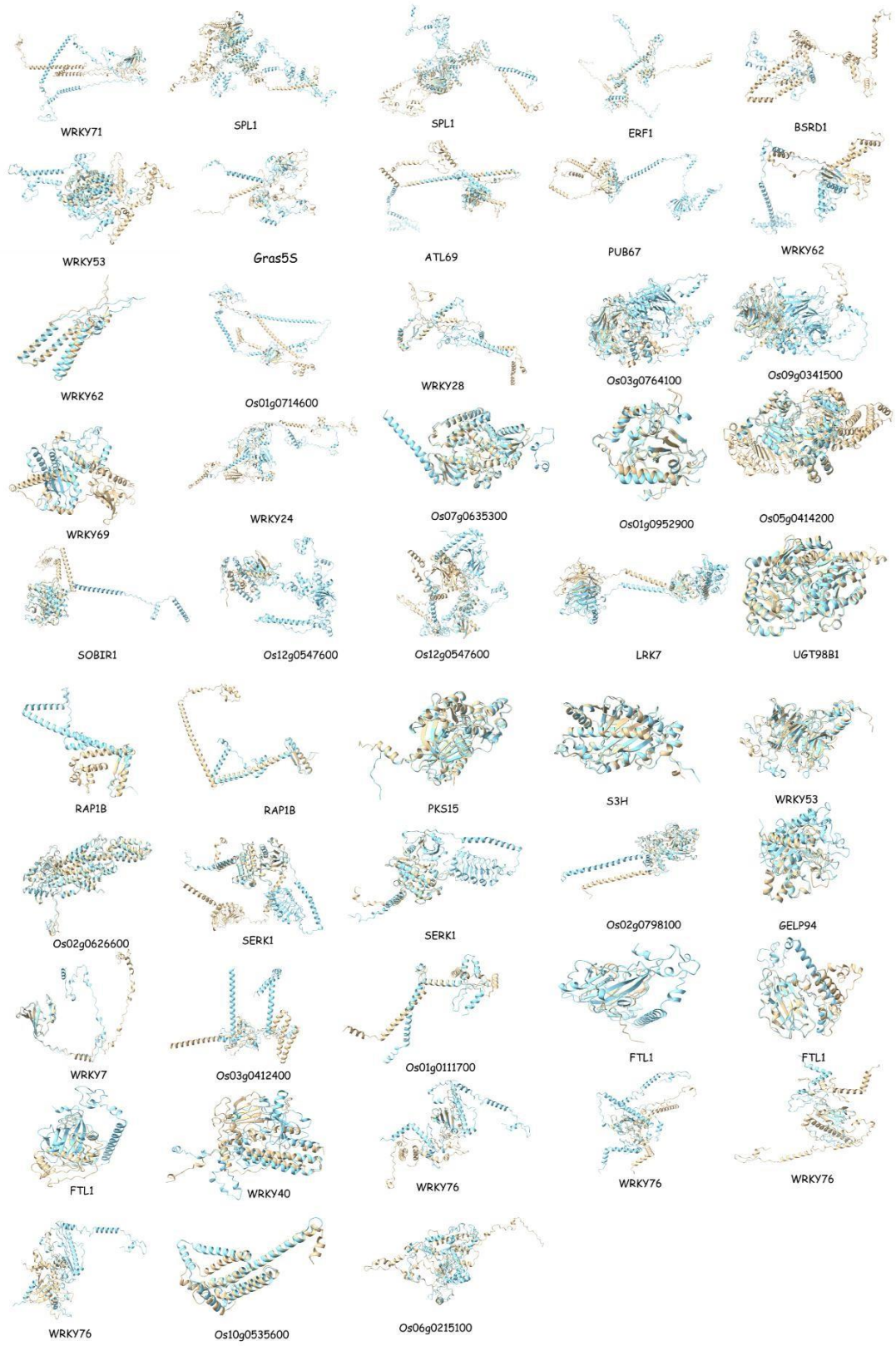

Fig 5. Protein structure prediction and superimposing for hub genes of *O.sativa* against *O.nivara*

Conclusion

Our investigation delves into the evolutionary trajectories and functional repertoires of WRKY transcription factors in wild rice species, illuminating their pivotal contributions to stress tolerance mechanisms. By elucidating regulatory networks and identifying key hub genes, our study presents valuable insights for targeted crop improvement strategies, essential for bolstering agricultural resilience in the face of environmental challenges. However, the present study doesn't involve experimental validation. Therefore, the next step would be to functionally characterize these genes and utilize the elite candidate genes in crop productivity through breeding programs or by deploying modern genome editing technologies.

Author Contributions

K.A. performed these experiments, analyzed the data, and prepared the first draft of the manuscript. S.M.S. and M.K.R edited the manuscript and helped in planning the layout of the work. All the authors have agreed on the current version of the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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