ISSN 2664-4169 (Print) & ISSN 2664-7923 (Online)
South Asian Research Journal of Biology and Applied Biosciences
Abbreviated Key Title: South Asian Res J Bio Appl Biosci

| Volume-8 | Issue-3 | May-Jun -2026 | **DOI: https://doi.org/10.36346/sarjbab.2026.v08i03.010**

Original Research Article

In Silico Genome-wide Identification of Salt Stress-Responsive Genomic Elements with Special Reference to WRKY Genes in *Vicia faba* L.

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Article History
Received: 20.04.2026
Accepted: 01.06.2026
Published: 13.06.2026

Abstract: *Vicia faba* (faba bean) is a globally significant cool-season grain legume valued for its high protein content, nitrogen-fixing capacity, and adaptability to diverse agro-climatic conditions. However, abiotic stresses, particularly soil salinization, severely constrain its productivity. Transcription factors of the WRKY superfamily play pivotal roles in regulating plant responses to abiotic stress, including salt tolerance. This study presents an integrated bioinformatics pipeline to identify, characterize, and analyze putative salt stress-responsive WRKY genes in *V. faba*. Using Arabidopsis thaliana WRKY8 (UniProt: Q9FL26) as a reference, we performed homology-based screening against the *V. faba* genome via Ensembl Plants BLAST. Candidate sequences underwent rigorous physicochemical profiling (ProtParam), conserved domain analysis (NCBI-CDD), motif elucidation (MEME Suite), phylogenetic reconstruction (MEGA), gene structure visualization (GSDS), and subcellular localization prediction (WoLF PSORT). Iterative filtering based on domain architecture and motif conservation yielded a high-confidence set of WRKY candidates. Phylogenetic analysis revealed diversification across Groups I, II, and III, with evidence of legume-specific expansion. The majority of candidates exhibited predicted nuclear localization, acidic to mildly basic isoelectric points, and thermostable aliphatic indices consistent with transcriptional regulatory functions. Gene structural analysis revealed intron-exon architectural diversity, suggesting evolutionary divergence and potential alternative splicing regulation. This work establishes a foundational genomic framework for understanding WRKY-mediated salt stress signaling in faba bean and identifies candidate targets for future functional validation and translational breeding toward salinity-tolerant cultivars.

Keywords: *Vicia faba, WRKY* transcription factors, salt stress, abiotic stress, bioinformatics, phylogenetics, conserved domains, subcellular localization.

INTRODUCTION

The faba bean (*Vicia faba* L.) represents one of the most important cool-season annual grain legumes cultivated worldwide. Historically regarded as an inexpensive yet high-quality vegetable protein source for both human consumption and animal feed, faba bean currently ranks among the top grain legumes globally (Gu *et al*., 2020). Worldwide production spans approximately 2.5 million hectares, yielding nearly 5 million tonnes annually, with China (36.7%), Ethiopia (20.1%), the United Kingdom (8.2%), and Australia (7.7%) dominating global output (FAO, 2018). The ecological versatility of *V. faba* distinguishes it from other major legumes. Unlike soybean, which performs poorly in cool climates, faba bean thrives in temperate and high-altitude environments with brief growing seasons (<100 days) (Huang *et al*., 2019; Stoddard and Hamalainen, 2011). It tolerates a broad pH range (6-9) and can produce acceptable yields in marginal soils where cereals such as barley and wheat struggle (Castanon *et al*., 1990; Etemadi *et al*., 2019). Moreover, faba bean exhibits exceptional biological nitrogen fixation efficiency among cool-season legumes, fixing between 50 and 330 kg N per hectare depending on climatic and management conditions (Galloway *et al*., 2004; Khazaei *et al*., 2019). This capacity reduces dependency on industrial nitrogen fertilizers, offering substantial ecosystem services that promote sustainable agricultural intensification.

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Abiotic stress encompasses all negative environmental factors that impede healthy plant development, including drought, salinity, extreme temperatures, nutrient deficiencies, and heavy metal toxicity (Fahad *et al*., 2017; Wu *et al*., 2023). These stressors disrupt cellular metabolism, morpho-physiological processes, and molecular signaling networks, ultimately manifesting as reduced growth, reproductive failure, and yield loss (Ahmad *et al*., 2023; Ben Rejeb *et al*., 2014). Soil salinization constitutes one of the most pervasive abiotic constraints to global agriculture. Approximately 900 million hectares of land, nearly one-third of irrigated agricultural area, are affected by elevated salt concentrations, with annual economic losses exceeding $27.3 billion (Qadir *et al*., 2014; Shahid *et al*., 2018). The problem is intensifying due to climate change, rising sea levels in coastal zones, and improper irrigation practices utilizing saline groundwater (Hailu and Mehari, 2021; Rengasamy, 2006). Saline soils are characterized by high concentrations of Na+, Cl-, Ca2+, SO4 2-, and HCO3-, with electrical conductivity (EC) exceeding 4 dS m-1 and exchangeable sodium percentage surpassing 15% (Osman, 2018). Salt stress imposes dual physiological challenges: osmotic stress, which reduces soil water potential and limits root water uptake, and ionic toxicity, whereby excessive Na+ and Cl- accumulation disrupts cellular metabolism, membrane integrity, and enzyme function (Flora *et al*., 2008; Van Zelm *et al*., 2020).

The WRKY transcription factor superfamily ranks among the largest and most functionally diverse groups of regulatory proteins in higher plants. The family name derives from its highly conserved 60-amino acid WRKY DNA-binding domain (DBD), which contains the invariant WRKYGQK heptapeptide at the N-terminus and a C-terminal zinc-finger motif (Eulgem *et al*., 2000; Rushton *et al*., 2010; Zhang and Wang, 2005). Based on domain architecture and zinc-finger configuration, WRKY proteins are classified into three principal groups. Group I members possess two WRKY DBDs and a C2H2 zinc-finger motif. Group II contains a single DBD with a C2H2 zinc finger and is further subdivided into five subgroups (IIa-IIe). Group III is distinguished by a single DBD with a C2HC zinc-finger motif (Bakshi and Oelmuller, 2014; Maeo *et al*., 2001). Functionally, WRKY TFs participate in virtually every aspect of plant life, regulating developmental processes, stress responses, and secondary metabolism. WRKY8 from Arabidopsis thaliana (UniProt: Q9FL26) has been mechanistically validated as a regulator of sodium and potassium homeostasis under salinity through antagonistic interaction with VQ9 and modulation of SOS (SALT OVERLY SENSITIVE) gene expression (Hu *et al*., 2013). Given the established role of WRKY8 in salt tolerance and the agricultural importance of faba bean, systematic identification and characterization of WRKY orthologs in *V. faba* represents a critical step toward understanding and potentially manipulating salt stress responses in this legume crop.

EXPERIMENTAL SECTION

This study employed an integrated computational biology pipeline to identify, characterize, and analyze salt stress-responsive WRKY genes in *Vicia faba*. The workflow proceeded sequentially through reference gene selection, homology-based ortholog retrieval, physicochemical characterization, conserved domain and motif analysis, phylogenetic reconstruction, gene structure visualization, subcellular localization prediction, and integrative data visualization.

Reference Gene Identification and Sequence Retrieval

The UniProt accession Q9FL26, encoding Arabidopsis thaliana WRKY8 transcription factor, was selected as the query reference based on its empirically validated role in ionic homeostasis under salinity. The complete amino acid sequence was retrieved in FASTA format from the UniProt database (The UniProt Consortium, 2023) after confirming the documented function of WRKY8 in antagonistic regulation with VQ9 and downstream modulation of SOS gene expression (Hu *et al*., 2013).

Homology Search and Ortholog Retrieval

The WRKY8 protein sequence was used as query in a BLAST search against the *V. faba* genome database via Ensembl Plants (Bolser *et al*., 2017). Significant hits were evaluated based on E-values, bit-scores, and alignment coverage. For each candidate gene, gene identifiers, chromosomal locations, base pair lengths, and complete amino acid sequences were retrieved. Coding sequence (CDS), genomic DNA, and protein sequence data were systematically downloaded and organized for downstream analyses.

Physicochemical Property Analysis

The physicochemical properties of all retrieved *V. faba* protein sequences were analyzed using the ProtParam tool on the ExPASy server (Gasteiger *et al*., 2005). Parameters computed included theoretical isoelectric point (pI), molecular weight, instability index, aliphatic index, and grand average of hydropathicity (GRAVY). These parameters provide insights into protein solubility, thermal stability, and hydrophobic nature under stress conditions.

Conserved Domain and Motif Analysis

Conserved domains were identified using the NCBI Conserved Domain Database (CDD) with Pfam hidden Markov models (Finn *et al*., 2016; Marchler-Bauer *et al*., 2017). Domain architecture was visualized using TBtools (Chen *et al*., 2020), and sequences lacking recognizable WRKY domains were excluded. De novo conserved motif discovery was

performed using the MEME Suite (Bailey *et al*., 2009), employing expectation-maximization algorithms to identify statistically significant sequence patterns. Candidates lacking at least two of three identified conserved motifs were subsequently filtered out.

Phylogenetic Reconstruction

Evolutionary relationships among filtered *V. faba* WRKY candidate proteins were reconstructed using MEGA version 12 (Tamura *et al*., 2021). Multiple sequence alignment was performed using the ClustalW algorithm (Thompson *et al*., 1994). Maximum Likelihood phylogenetic trees were constructed using the JTT+G+I substitution model with 1000 bootstrap replicates. Trees were exported in Newick format and annotated using iTOL (Letunic and Bork, 2021) for publication-quality visualization with branch coloring by evolutionary clade.

Gene Structure and Subcellular Localization Analysis

Intron-exon organization was analyzed using the GSDS server (Guo *et al*., 2007; Hu *et al*., 2015) with CDS and corresponding genomic DNA sequences as inputs. Subcellular localization was predicted using WoLF PSORT (Horton *et al*., 2007) in plant mode, generating probability scores for nuclear, cytoplasmic, mitochondrial, chloroplast, Golgi, plasma membrane, and extracellular compartments. Localization data were compiled and visualized as comparative heatmaps using TBtools.

RESULTS AND DISCUSSION

The integrated bioinformatics pipeline successfully identified, characterized, and analyzed a high-confidence set of salt stress-responsive WRKY transcription factor candidates in *Vicia faba*. The results are presented sequentially, with integrated discussion of their biological implications.

Reference Gene Functional Annotation

UniProt entry Q9FL26 was confirmed to encode WRKY8, a 269-amino acid transcription factor containing one WRKY DNA-binding domain (Group II architecture). Its established role in regulating sodium and potassium homeostasis through antagonistic interaction with VQ9 and downstream modulation of SOS genes provided a functionally validated anchor for homology-based screening. The selection of this reference was strategically motivated by its dual regulatory capacity: WRKY8 functions not merely as an activator but as a molecular rheostat that both promotes stress-ameliorative genes and represses negative regulators (Hu *et al*., 2013). This nuanced regulatory logic increases the probability that BLAST-derived homologs in *V. faba* share not only sequence ancestry but potentially analogous physiological functions.

Identification of Putative Orthologs in *Vicia faba*

BLAST analysis of the WRKY8 query against the *V. faba* genome yielded multiple significant hits with high sequence similarity and low E-values. The retrieval of numerous candidates was notable given the historically challenging genomic landscape of faba bean, characterized by an exceptionally large (~13 Gb), highly repetitive genome that has only recently yielded chromosome-scale assemblies (Jayakodi *et al*., 2023). The abundance of hits suggests lineage-specific expansion of WRKY genes in faba bean, consistent with patterns observed in other legume species where abiotic stress-associated transcription factor families frequently amplify via tandem duplication or whole-genome duplication (Jiang *et al*., 2017). However, BLAST similarity does not guarantee functional orthology. We therefore treated initial hits as candidate sequences requiring iterative validation through domain, motif, and phylogenetic analyses.

Physicochemical Profiles as Functional Readouts

ProtParam analysis revealed distinct molecular phenotypes across the candidate set. Theoretical isoelectric points (pI) ranged from acidic (~5.0) to mildly basic (~8.5), with a modal distribution around pH 6.5-7.5. This charge distribution is compatible with nuclear function, as transcription factors must maintain solubility in the mildly alkaline nucleoplasm while engaging in electrostatic interactions with DNA phosphate backbones. Molecular weights varied considerably, reflecting differences in N-terminal and C-terminal extensions beyond the conserved WRKY domain. Notably, several candidates scored marginally above the canonical instability threshold of 40. Rather than indicating structural deficiency, controlled instability may serve as a regulatory feature: stress-responsive transcription factors often require rapid proteolytic clearance to prevent constitutive pathway activation, and an inherently labile fold could facilitate ubiquitin-mediated degradation (Matsushita *et al*., 2013; Miao and Zentgraf, 2010). The uniformly negative GRAVY scores and moderate-to-high aliphatic indices sketch a molecular phenotype of hydrophilic, thermostable regulatory proteins adapted to dynamic nuclear environments.

Domain Validation and Architectural Integrity

NCBI-CDD analysis using Pfam hidden Markov models identified the diagnostic WRKY DNA-binding domain in the majority of candidate sequences. The domain spanned approximately 50-60 amino acids and contained the invariant WRKYGQK heptapeptide followed by expected C2H2 or C2HC zinc-finger configurations. Group I candidates exhibited

two WRKY domains, while Groups II and III possessed single domains with distinct zinc-coordination chemistries. Domain E-values were highly significant (typically 10-20 to 10-40), confirming biological authenticity. Several candidates contained additional ancillary domains, including leucine zipper motifs that may mediate dimerization with other stress-responsive factors. Sequences lacking recognizable domains were excluded to prevent contamination by pseudogenes, assembly artifacts, or non-specific BLAST matches. This conservative filtering step was essential for maintaining phylogenetic resolution and functional credibility.

Conserved Motif Discovery and Functional Inference

While Pfam identifies established domains, the MEME Suite uncovered lineage-specific motifs that may represent recently evolved functional elements. Three statistically significant conserved motifs were identified with low E-values and high information content. Motif 1 localized to the N-terminal region and exhibited similarity to known transcriptional activation domains. Motif 2 mapped proximal to the WRKY domain and potentially represents a nuclear localization signal or protein-protein interaction interface. Motif 3 occupied a more variable C-terminal position and, while lacking direct InterPro homology, is evolutionarily conserved across *V. faba* paralogs, implying purifying selection. An additional filtering step retained only candidates possessing at least two of three motifs, ensuring that the final dataset comprised multifunctional proteins with robust structural and functional signatures rather than domain-only minimalists.

Phylogenetic Diversification and Evolutionary Implications

Maximum Likelihood phylogenetic reconstruction classified the filtered *V. faba* WRKY candidates into distinct evolutionary clades corresponding to Groups I, II (subgroups IIa-IIe), and III. Group I formed a well-supported monophyletic clade characterized by dual WRKY domains. Group II was the largest and most structurally heterogeneous clade, reflecting extensive diversification. Notably, Group III appeared expanded relative to Arabidopsis, suggesting legume-specific retention or independent duplication events. Group III members, distinguished by C2HC zinc fingers, have been implicated in diverse stress responses and senescence regulation; their expansion may reflect adaptive evolution to the cool-season, high-stress environments where faba bean is cultivated. Bootstrap support values exceeding 80% confirmed the robustness of major branching patterns, permitting confident assignment of candidates to evolutionary groups and guiding the selection of representative genes for future functional assays.

Gene Structure as an Evolutionary and Regulatory Archive

GSDS analysis revealed substantial variation in intron-exon architecture. Some genes displayed compact structures with minimal intronic content (2-3 exons), resembling ancestral WRKY configurations, while others exhibited complex architectures with up to five introns and variable exon lengths. Intron phase analysis revealed a predominance of phase-0 introns (between codons), which are evolutionarily conserved and minimally disruptive to reading frames. Phylogenetic clades exhibited conserved gene structure patterns: Group I members consistently contained four introns, whereas Group III genes were predominantly intron-poor. This structural diversity suggests evolutionary divergence through intron loss, gain, and alternative splicing that may fine-tune stress-responsive expression. Introns are not silent spacers; they harbor cis-regulatory elements, stress-responsive enhancers, and epigenetic modification landmarks that modulate transcriptional output. The structural diversity documented here therefore represents potentially functional variation.

Subcellular Geography and Regulatory Dynamics

WoLF PSORT analysis predicted nuclear localization as the primary compartment for the majority of candidates, with probability scores frequently exceeding the 80% reliability threshold. This aligns with the canonical function of WRKY proteins as transcriptional regulators binding chromatin-localized W-box elements. However, several candidates exhibited non-negligible cytoplasmic probabilities. Nucleocytoplasmic partitioning is a documented regulatory mechanism for transcription factors; cytoplasmic sequestration could maintain WRKY proteins in an inactive reservoir until stress-triggered signaling cascades facilitate nuclear import. One Group III member received nearly equal nuclear and mitochondrial scores, warranting investigation into potential organellar roles given emerging evidence of organellar transcriptional regulation (Van Aken *et al*., 2013; Vanderauwera *et al*., 2012). These predictions, while requiring experimental validation through fluorescent protein fusions, orient future research toward potentially novel regulatory nodes.

Integrated Structural and Evolutionary Visualization

TBtools integration of phylogenetic, domain, and motif data revealed strong co-linearity between evolutionary relationships and molecular architecture. Members of the same phylogenetic clade consistently shared identical domain arrangements and motif compositions, indicating that duplication events preserved functional modules intact. Conversely, basal lineages exhibited chimeric or divergent architectures, potentially representing evolutionary intermediates or neofunctionalized derivatives. The iTOL-annotated phylogenetic tree provided a publication-quality visual narrative, with branch coloring by WRKY group membership and concentric metadata rings displaying domain presence, motif counts,

and localization predictions. This multi-layered approach transformed the tree from a mere branching diagram into an information-dense dashboard suitable for guiding targeted functional assays.

Limitations and Future Perspectives

This bioinformatics pipeline was deliberately conservative, prioritizing specificity over sensitivity. Nevertheless, several limitations must be acknowledged. In silico predictions remain theoretical until validated by gene expression profiling, yeast one-hybrid assays, and functional complementation in Arabidopsis wrky mutants. The absence of publicly available *V. faba* salt stress transcriptome data precluded direct correlation of our candidate list with NaCl-induced expression changes. Moreover, reliance on a draft genome assembly means that true WRKY paralogs residing in repetitive or centromeric regions may be missing or collapsed. Despite these constraints, the dataset offers immediate translational utility. The phylogenetic framework identifies expanded and contracted groups relative to model species, guiding targeted functional assays. The WRKY8-centered orthology network provides a rational starting point for CRISPR-based genome editing or transgenic approaches to enhance salt tolerance in faba bean (Jayakodi *et al*., 2023). As global soil salinization accelerates under climate change, such molecular insights are essential for safeguarding the productivity of this nutritionally and environmentally critical legume crop.

CONCLUSION

This study presents a comprehensive bioinformatics characterization of salt stress-responsive WRKY transcription factor candidates in *Vicia faba*. Through an integrated pipeline combining homology search, physicochemical profiling, domain and motif analysis, phylogenetic reconstruction, gene structure visualization, and subcellular localization prediction, we identified and curated a high-confidence set of WRKY genes structurally and evolutionarily competent to participate in salt stress signaling. The candidates exhibit canonical WRKY domain architectures, conserved regulatory motifs, predicted nuclear localization, and phylogenetic diversification across Groups I, II, and III. Notably, Group III appears expanded in faba bean, suggesting legume-specific evolutionary adaptation. Gene structural diversity indicates potential for complex transcriptional and post-transcriptional regulation through alternative splicing and intron-mediated regulatory elements. These findings establish a foundational genomic resource for understanding WRKY-mediated abiotic stress responses in faba bean and identify promising candidate genes for downstream functional validation, marker-assisted selection, and genome editing aimed at developing salinity-tolerant cultivars. As global soil salinization accelerates under climate change, such molecular insights are essential for safeguarding the productivity of this nutritionally and environmentally critical legume crop.

Acknowledgements

The authors gratefully acknowledge the computational resources, lab facilities of the Dept. of Bioinformatics, JV College Baraut, used for the current research work.

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