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Original Research Article

In Silico Genome-wide Identification of Salt Stress-Responsive Genomic Elements with Special Reference to RD22 Genes in *Vigna Unguiculata* L.

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Abstract: *Vigna Unguiculata* (cowpea) is a globally significant tropical legume valued for its high protein content, drought tolerance, and adaptability to marginal agro-climatic conditions. However, abiotic stresses, particularly soil salinization, severely constrain its productivity in arid and semi-arid regions. The RD22 (Responsive to Dehydration 22) gene family, encoding BURP domain-containing proteins, plays pivotal roles in regulating plant responses to abiotic stress, including salt and drought tolerance. This study presents an integrated bioinformatics pipeline to identify, characterize, and analyze putative salt stress-responsive RD22 genes in *V. Unguiculata*. Using Arabidopsis thaliana RD22 (UniProt: P22247) as a reference, we performed homology-based screening against the *V. Unguiculata* 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 RD22 candidates. Phylogenetic analysis revealed diversification across the RD22-like subfamily, with evidence of legume-specific expansion. The majority of candidates exhibited predicted apoplastic and vacuolar localization, acidic to mildly basic isoelectric points, and thermostable aliphatic indices consistent with stress-responsive 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 RD22-mediated salt stress signaling in cowpea and identifies candidate targets for future functional validation and translational breeding toward salinity-tolerant cultivars.

Keywords: *Vigna Unguiculata*, RD22 Genes, BURP Domain, Salt Stress, Abiotic Stress, Phylogenetics, Conserved Domains, Subcellular Localization.

INTRODUCTION

Cowpea (*Vigna Unguiculata* L. Walp.; Fabales: Fabaceae) represents one of the most important tropical grain legumes cultivated worldwide, particularly in sub-Saharan Africa, Asia, and parts of the Americas. Historically regarded as a resilient crop adapted to hot and dry environments, cowpea currently serves as a critical source of dietary protein for millions of people in developing nations (Singh *et al*., 2003). Worldwide production spans approximately 15 million hectares, yielding more than 8.8 million tonnes annually, with Africa occupying more than 95% of the cultivated area, particularly the arid and semi-arid regions of West Africa (Fatokun *et al*., 2018). The ecological versatility of *V. Unguiculata* distinguishes it from other major legumes. Unlike soybean, which performs poorly in hot and dry climates, cowpea thrives in environments with limited rainfall and high temperatures, making it an ideal crop for sustainable agriculture in marginal lands (Hall, 2004; Ehlers and Hall, 1997). It tolerates a broad range of soil conditions and can produce acceptable yields in sandy soils where other crops struggle (Boukar *et al*., 2018). Moreover, cowpea exhibits exceptional nitrogen fixation efficiency, contributing between 50 and 200 kg N per hectare depending on climatic and management conditions (Sanginga *et al*., 2000). 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+, SO42-, 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). Cowpea, despite its inherent drought tolerance, shows significant sensitivity to salt stress during the vegetative stage, with seed yield reduced by 50% at moderate salinity levels (Maas and Poss, 1989).

The RD22 (Responsive to Dehydration 22) gene family encodes members of the BURP (BNM2, USP, RD22, PG1b) domain-containing protein superfamily, a group of plant-specific proteins that play critical roles in stress responses and developmental processes. The BURP domain, typically located at the C-terminus of these proteins, is characterized by a highly conserved sequence of approximately 230 amino acids containing four conserved cysteine residues and several other invariant amino acids (Hattori *et al*., 1998; Granger *et al*., 2002). Based on domain architecture and sequence similarity, BURP proteins are classified into four principal subfamilies: BNM2-like, USP-like, RD22-like, and PG1b-like (Wang et al., 2015). The RD22-like subfamily has been particularly associated with abiotic stress responses. RD22 from Arabidopsis thaliana (UniProt: P22247) was originally identified as a gene strongly induced by dehydration, salt stress, and exogenous abscisic acid (ABA) application (Yamaguchi-Shinozaki and Shinozaki, 1993). The protein sequence of AtRD22 exhibits considerable homology to the unknown seed protein (USP) of Vicia faba, and its expression is mediated by ABA-dependent signaling pathways requiring protein synthesis (Yamaguchi-Shinozaki and Shinozaki, 1993). Functional studies have demonstrated that RD22 acts as a suppressor of the ABA-mediated moisture stress response, with loss-of-function mutants exhibiting enhanced drought tolerance (Alexander and Grierson, 2002). The heterologous expression of soybean GmRD22 in Arabidopsis and rice has been shown to enhance salinity stress tolerance, possibly through increasing lignin production and apoplastic peroxidase activity (Wang *et al*., 2012). Given the established role of RD22 in salt tolerance and the agricultural importance of cowpea, systematic identification and characterization of RD22 orthologs in *V. Unguiculata* 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 RD22 genes in *Vigna Unguiculata*. 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 P22247, encoding Arabidopsis thaliana RD22 dehydration-responsive protein, was selected as the query reference based on its empirically validated role in dehydration and salt stress responses. The complete amino acid sequence was retrieved in FASTA format from the UniProt database (The UniProt Consortium, 2023) after confirming the documented function of RD22 in ABA-mediated stress responses and its characterization as a BURP domain-containing protein (Yamaguchi-Shinozaki and Shinozaki, 1993).

Homology Search and Ortholog Retrieval

The RD22 protein sequence was used as query in a BLAST search against the *V. Unguiculata* 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. Unguiculata* 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 BURP 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. Unguiculata* RD22 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 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, extracellular, and vacuolar 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 RD22 transcription factor candidates in *Vigna Unguiculata*. The results are presented sequentially, with integrated discussion of their biological implications.

Reference Gene Functional Annotation

UniProt entry P22247 was confirmed to encode RD22, a 189-amino acid dehydration-responsive protein containing one BURP domain at the C-terminus (RD22-like subfamily architecture). Its established role in regulating dehydration and salt stress responses through ABA-mediated signaling pathways provided a functionally validated anchor for homology-based screening. The selection of this reference was strategically motivated by its dual regulatory capacity: RD22 functions not merely as an activator but as a molecular modulator that both responds to stress signals and participates in cell wall modification and lignin biosynthesis (Wang *et al*., 2012; Alexander and Grierson, 2002). This nuanced regulatory logic increases the probability that BLAST-derived homologs in *V. Unguiculata* share not only sequence ancestry but potentially analogous physiological functions.

Identification of Putative Orthologs in *Vigna Unguiculata*

BLAST analysis of the RD22 query against the *V. Unguiculata* genome yielded multiple significant hits with high sequence similarity and low E-values. The retrieval of numerous candidates was notable given the recently available chromosome-scale genome assembly of cowpea (Lonardi *et al*., 2019). The abundance of hits suggests lineage-specific expansion of RD22 genes in cowpea, consistent with patterns observed in other legume species where abiotic stress-associated gene 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 (~5.2) to mildly basic (~8.0), with a modal distribution around pH 6.0-7.0. This charge distribution is compatible with apoplastic and vacuolar function, as BURP proteins must maintain solubility in the acidic apoplast while engaging in cell wall modification processes. Molecular weights varied considerably, reflecting differences in N-terminal extensions beyond the conserved BURP 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 proteins 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 extracellular environments.

Domain Validation and Architectural Integrity

NCBI-CDD analysis using Pfam hidden Markov models identified the diagnostic BURP domain in the majority of candidate sequences. The domain spanned approximately 200-230 amino acids and contained the characteristic four conserved cysteine residues and other invariant amino acids typical of the BURP superfamily. Domain E-values were highly

significant (typically 10^-15 to 10^-35), confirming biological authenticity. Several candidates contained additional ancillary motifs, including signal peptides at the N-terminus that may direct secretion to the apoplast. Sequences lacking recognizable BURP 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 signal peptide and proline-rich domains. Motif 2 mapped to the central region and potentially represents a cell wall interaction interface or protein-protein interaction domain. Motif 3 occupied the C-terminal BURP domain position and contained the characteristic cysteine-rich motif essential for protein folding and stability. 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. Unguiculata* RD22 candidates into distinct evolutionary clades corresponding to the RD22-like subfamily and related BURP groups. The RD22-like clade was the largest and most structurally heterogeneous, reflecting extensive diversification. Notably, the RD22-like clade appeared expanded relative to Arabidopsis, suggesting legume-specific retention or independent duplication events. 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 BURP configurations, while others exhibited complex architectures with up to four 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: RD22-like members consistently contained three introns, resembling the Arabidopsis RD22 gene structure (Yamaguchi-Shinozaki and Shinozaki, 1993). 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 apoplastic and vacuolar localization as the primary compartments for the majority of candidates, with probability scores frequently exceeding the 70% reliability threshold. This aligns with the canonical function of RD22 proteins as extracellular regulators involved in cell wall modification and stress signaling. However, several candidates exhibited non-negligible cytoplasmic and nuclear probabilities. Nucleocytoplasmic partitioning is a documented regulatory mechanism for stress-responsive proteins; cytoplasmic sequestration could maintain RD22 proteins in an inactive reservoir until stress-triggered signaling cascades facilitate secretion or nuclear import. One candidate received nearly equal apoplastic and vacuolar scores, warranting investigation into potential dual localization given emerging evidence of vacuolar stress-responsive protein storage (Wang *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 RD22 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, quantitative RT-PCR, and functional complementation in Arabidopsis rd22

mutants. The absence of publicly available *V. Unguiculata* 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 RD22 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 RD22-centered orthology network provides a rational starting point for CRISPR-based genome editing or transgenic approaches to enhance salt tolerance in cowpea (Boukar *et al*., 2018). 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 RD22 gene candidates in. 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 RD22 genes structurally and evolutionarily competent to participate in salt stress signaling. The candidates exhibit canonical BURP domain architectures, conserved regulatory motifs, predicted apoplastic and vacuolar localization, and phylogenetic diversification within the RD22-like subfamily. Notably, the RD22-like clade appears expanded in cowpea, 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 RD22-mediated abiotic stress responses in cowpea 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.

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