International Journal on Science and Technology

One Day National Seminar on

"Advances in Life Sciences for Diversity, Applications, and Human Welfare"

(ALSDAHW – 2025)

Organized by Department of Microbiology & Department of Biotechnology

M.V.S. Government Arts & Science College (Autonomous), Mahabubnagar-509001

E-ISSN: 2229-7677 • Website: www.ijsat.org • Email: editor@ijsat.org

In-Silico Prediction of miRNA Drought Stress-Responsive Genes in *Gymnema sylvestre*

Madhinni Sahitya[1], Syeda Amena Kausar[2]

[1,2]Department of Genetics and Biotechnology, Veeranari Chakali Ilamma Women's University, Koti, Hyderabad, Telangana – 500 001, India.

Abstract

Water limitation poses a formidable challenge to plant vitality, growth vigor, and harvest yields, compelling plants to orchestrate multilayered genetic defenses. Among these safeguards, microRNAs (miRNAs)—brief non-coding RNA entities—wield precise control over stress adaptation by dismantling or muting target transcripts during crisis. While drought response pathways have been extensively studied in major crop species such as rice and wheat, comparable regulatory mechanisms remain poorly understood in *Gymnema sylvestre*, a medicinal plant widely valued for its antidiabetic properties, largely due to limited genomic information. In this study, an in-silico approach was used to predict miRNAs associated with drought stress responses in *G. sylvestre*. Key drought-related genes, including peroxidases, heat shock proteins, NAC, WRKY and MYB transcription factors, aquaporins, and protein kinases, were identified from published de novo transcriptome datasets. Owing to the limited availability of *G. sylvestre* gene sequences, homologous reference mRNAs from model plant species were retrieved from the NCBI database and analyzed using the psRNATarget tool with standard plant miRNA libraries. Predicted miRNA–mRNA interactions were filtered based on binding strength (expectation ≤ 3.0) and mode of inhibition. The analysis identified several conserved miRNAs potentially regulating genes involved in oxidative stress defense, protein protection, transcriptional regulation, water transport, and stress signaling under drought conditions. These predicted regulatory networks provide initial insights into miRNA-mediated drought responses in *G. sylvestre* and establish a foundation for future experimental validation. The findings contribute to understanding stress tolerance mechanisms in medicinal plants and may support conservation and climate-resilience strategies under changing environmental conditions.

Keywords: *Gymnema sylvestre*; miRNA; drought tolerance; psRNATarget; NAC; WRKY.

1. Introduction

Drought stress represents one of the most critical challenges to global food security and agricultural sustainability, imposing severe constraints on plant growth, development, and yield. As climate change exacerbates the frequency and intensity of water deficit, understanding the molecular mechanisms

underpinning plant drought tolerance has become a research imperative. In response to drought, plants activate a complex, multilayered adaptation program involving physiological adjustments, hormonal signaling—most notably abscisic acid (ABA)—and the extensive transcriptional reprogramming of stress-responsive genes (Mukherjee et al., 2023). Beyond this transcriptional control, post-transcriptional regulation, particularly by microRNAs (miRNAs), has emerged as a crucial layer for the rapid and precise fine-tuning of gene expression essential for survival under transient or prolonged stress (Raza et al., 2023). miRNAs are small, endogenous, non-coding RNAs (approximately 20-24 nucleotides) that function as key negative regulators of gene expression by guiding the sequence-specific cleavage or translational inhibition of target messenger RNAs (mRNAs). This mechanism allows for the swift modulation of protein levels, making miRNAs ideal master regulators for dynamic stress responses (Luo et al., 2025). Recent research has consolidated the understanding that conserved miRNA families are integral to drought adaptation networks across diverse plant species. They orchestrate critical physiological adjustments, including stomatal closure, osmotic balance, reactive oxygen species (ROS) scavenging, and root architecture remodeling. For instance, the miR169 family, which targets Nuclear Factor Y (NF-Y) transcription factors, is consistently downregulated under drought to enhance the expression of NF-YA subunits, thereby improving stomatal regulation and stress tolerance, as demonstrated in tomato and alfalfa (Ji et al., 2023). Similarly, miR164-mediated regulation of NAC transcription factors influences root development and senescence pathways, which are vital for water uptake and resource allocation during stress (Wang et al., 2025).

The discovery and functional characterization of drought-responsive miRNAs continue to expand. Recent studies highlight specific miRNAs as central players. Yin et al. (2018) reported that drought-induced miR319 targets TCP transcription factors, affecting jasmonic acid biosynthesis and leaf morphology to reduce water loss. Furthermore, the miR398 family's role in modulating copper/zinc superoxide dismutase (CSD) levels illustrates a conserved strategy to reconfigure the antioxidant system under oxidative stress triggered by drought (Suzuki et al., 2019). These examples underscore a sophisticated regulatory paradigm where miRNAs act as molecular switches, fine-tuning the expression of both transcription factors and effector proteins to optimize the balance between stress defense and growth (Yan et al., 2025).

Despite this advanced understanding in model and staple crop species, the landscape of miRNA-mediated drought response remains almost entirely unexplored in medicinal plants. These species possess immense pharmacological value but are frequently neglected in genomic studies, creating a significant knowledge gap that hinders their conservation and resilient cultivation. *Gymnema sylvestre* (Retz.) Schult., commonly known as Gurmar, is a prime example. This perennial climber is renowned in traditional medicine for its potent anti-diabetic properties, attributed to bioactive compounds such as gymnemic acids. Its sustainable cultivation is increasingly threatened by abiotic stresses like drought (Preetha et al., 2025). While foundational genomic resources, including de novo transcriptome assemblies, have been developed, these studies have primarily focused on elucidating pathways for secondary metabolite biosynthesis (Kalariya et al., 2024). However, a systematic understanding of post-transcriptional regulatory networks controlling

abiotic stress responses in *Gymnema sylvestre* is still missing, prompting the need for the present investigation.

However, a systematic understanding of post-transcriptional regulatory networks controlling abiotic stress responses in *Gymnema sylvestre* is still missing, prompting the need for the present investigation. This challenge is further compounded by the limited availability of genomic resources for this non-model medicinal plant, which restricts the application of conventional experimental approaches. Under such constraints, in silico predictive strategies have emerged as indispensable tools for uncovering regulatory relationships and generating biologically meaningful hypotheses. Computational platforms such as the psRNATarget server enable the prediction of miRNA binding sites on homologous mRNA sequences, providing a feasible framework to infer post-transcriptional regulation in species lacking complete genome assemblies (Dai et al., 2018). Integrating these predictions with network-based analyses allows the identification of key regulatory miRNAs and central hub genes, thereby prioritizing candidates for subsequent experimental validation and reducing the cost and complexity of wet-lab investigations (Karimipour et al., 2025).

Accordingly, this study adopts an integrated in silico pipeline to predict and characterize the miRNA–mRNA interaction network associated with drought stress responses in *G. sylvestre*. The analysis focuses on major drought-responsive gene families derived from available transcriptomic datasets, including antioxidant enzymes (peroxidases), molecular chaperones (heat shock proteins), signal transducers (protein kinases), water transporters (aquaporins), and key transcription factor families such as NAC, MYB, and WRKY. The specific objectives are to: (1) identify core drought-responsive genes from published *G. sylvestre* transcriptomic data; (2) predict conserved miRNAs targeting these genes using homology-based bioinformatics; (3) construct and visualize the resultant miRNA-mRNA interaction network; and (4) identify key hub genes and regulatory miRNAs through network topology analysis. The findings will generate a prioritized set of mechanistic hypotheses, establishing a crucial foundation for future experimental validation through techniques like qRT-PCR, degradome sequencing, and transgenic studies. Ultimately, this work aims to contribute to understanding and enhancing drought tolerance in this valuable medicinal species, supporting its resilience and sustainable utilization.

2. Methodology

2.1 Identification of Drought-Responsive Genes from Transcriptomic Data

Candidate drought-responsive genes from *Gymnema sylvestre* were identified through a systematic literature mining approach. Published research articles and associated supplementary data containing de novo transcriptome assemblies of *G. sylvestre* were sourced using academic search engines including Google Scholar, PubMed, and institutional library databases. The search keywords included "*Gymnema sylvestre* transcriptome," "de novo assembly," and "stress-responsive genes." From the identified transcriptome studies, particularly the work by, comprehensive lists of annotated transcripts were obtained. Manual curation was performed on the annotated transcript tables to select genes with established functional roles in drought stress responses in plants, a strategy commonly employed for non-model

species (Ayachit et al., 2019). The selection criteria were based on best-hit BLAST annotations against the NCBI non-redundant (nr) protein database. Representative genes from the following key functional categories were prioritized, based on their well-documented centrality in drought adaptation networks. Antioxidant Defense: Peroxidases (POD); 2) Protein Stability and Folding: Heat Shock Proteins (HSPs); 3) Transcriptional Regulation: NAC, MYB, and WRKY family transcription factors. Water Transport: Aquaporins (PIPs); 5) Signal Transduction: Protein Kinases (PK). The corresponding transcript IDs, gene/protein names, best-hit annotations, and inferred functional relevance were compiled into a structured database consolidated table.

2.2. Retrieval of Reference mRNA Sequences

Due to the absence of a fully sequenced and annotated genome for *G. sylvestre*, a homology-based strategy was employed, a standard and validated approach for comparative genomics in non-model medicinal plants (Ayachit et al., 2019). The selected gene names and their best-hit annotations from the transcriptome data were used as queries to search the National Center for Biotechnology Information (NCBI) Nucleotide database. To ensure accuracy and reliability, well-annotated, full-length mRNA sequences from model and closely related plant species (Arabidopsis thaliana, Catharanthus roseus) were retrieved. For each target gene, the most representative and complete mRNA sequence (in FASTA format) was downloaded for subsequent analysis. This step was critical to provide a reliable template for miRNA target prediction using established plant miRNA libraries.

2.3. In-Silico Prediction of miRNA Targets

The prediction of miRNAs targeting the retrieved reference mRNA sequences was performed using the **psRNATarget** web server with its 2017 release parameters. This server is specifically designed for plant small RNA target analysis and employs a sequential workflow that evaluates target site accessibility and complementary pairing, making it a widely used tool for such exploratory studies (Dai et al., 2018). The reference mRNA sequences in FASTA format were submitted as "Target Sequences." The "Small RNA Libraries" option was set to "Plant (miRBase)" to search against all known plant miRNAs.

The analysis was executed with default stringent parameters: Expectation score cut-off: 3.0, and the scoring schema V2 (2017 release). The expectation (E) score represents the number of predicted targets expected by chance; a lower score indicates higher prediction confidence. Two modes of inhibition were considered: Cleavage (predominant in plants) and Translation inhibition. The server's output provides detailed information, including the miRNA name, target gene, expectation score, inhibition mode, and the precise binding position (UPE) on the target mRNA. All predicted interactions with an expectation score ≤ 3.0 were retained for further analysis.

2.4. Construction and Analysis of miRNA-mRNA Interaction Networks

The filtered list of miRNA-mRNA interaction pairs was used to construct a regulatory network. The open-source software **Cytoscape version 3.9.1** was utilized for network visualization and analysis, a standard platform for integrating biomolecular interaction data. Two node types were defined: "miRNA" and "mRNA." A directed edge was drawn from miRNA node to an mRNA node, representing a predicted regulatory interaction.

International Journal on Science and Technology
One Day National Seminar on
"Advances in Life Sciences for Diversity, Applications, and Human Welfare"
(ALSDAHW – 2025)
Organized by Department of Microbiology & Department of Biotechnology
M.V.S. Government Arts & Science College (Autonomous), Mahabubnagar-509001
E-ISSN: 2229-7677 • Website: www.ijsat.org • Email: editor@ijsat.org

To identify central regulatory players within the network, a hub analysis was performed using the **cytoHubba** plugin (Chin et al., 2014). The "Degree" centrality method was applied, which ranks nodes based on the number of connections (edges) they possess. Nodes with a high degree are considered hubs and are potentially crucial for the stability and functionality of the regulatory network, an approach effectively used in recent plant stress network studies. The top 10 hubs were identified and their scores recorded (Razalli et al., 2025).

2.5. Data Standardization and Representation

All raw results from the psRNATarget analysis were compiled, standardized, and filtered based on the expectation score threshold. The finalized data is presented in comprehensive tables within the results section. Network visuals generated by Cytoscape were exported as high-resolution images for publication. The overall in-silico workflow adopted in this study is illustrated in Figure 1.

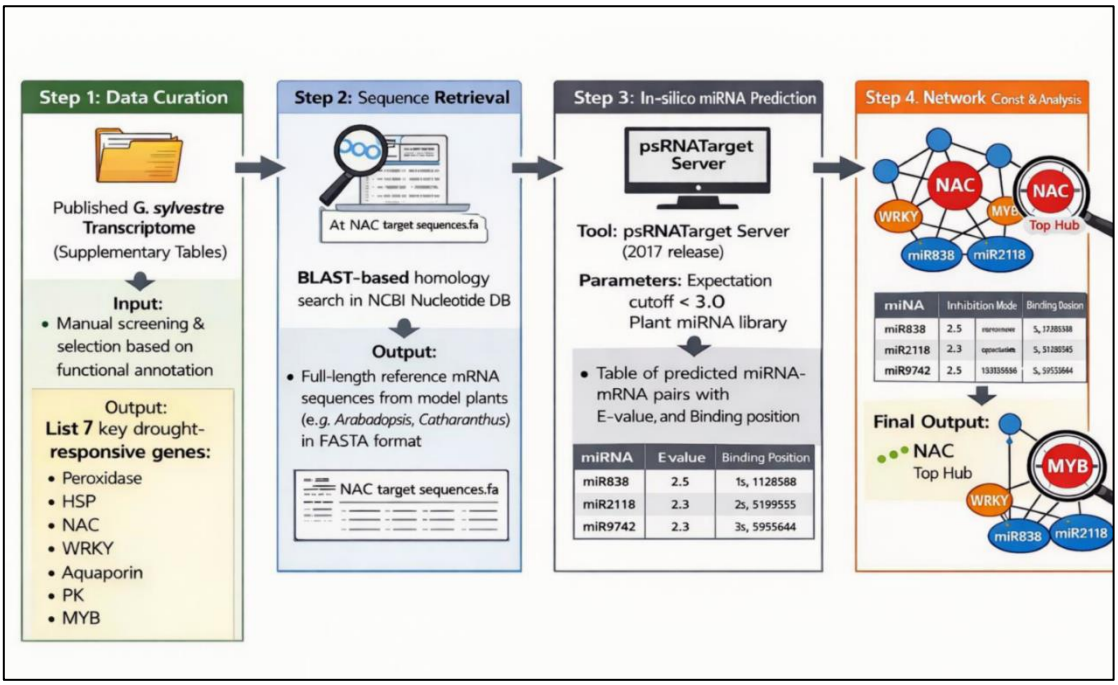

Figure 1. In-silico workflow for miRNA target prediction and network analysis in *Gymnema sylvestre*

3. Results

3.1. Identification of Core Drought-Responsive Genes

From the manual curation of the *G. sylvestre* de novo transcriptome data, seven key transcripts representing major drought-responsive functional categories were selected for in-depth analysis. The details of these transcripts, including their IDs, best-hit annotations, and functional relevance, are summarized in Table 1.

International Journal on Science and Technology
One Day National Seminar on
"Advances in Life Sciences for Diversity, Applications, and Human Welfare"
(ALSDAHW – 2025)
Organized by Department of Microbiology & Department of Biotechnology
M.V.S. Government Arts & Science College (Autonomous), Mahabubnagar-509001
E-ISSN: 2229-7677 • Website: www.ijsat.org • Email: editor@ijsat.org

Table 1: Selected drought stress–responsive genes identified from de novo transcriptome analysis of *Gymnema sylvestre*.

S.No.	Transcript ID	Gene / Protein	Best-hit Annotation	Functional Relevance
1	TRINITY_DN1880_c0_g1_i1	Peroxidase	Peroxidase activity	Oxidative stress scavenging
2	TRINITY_DN1694_c0_g1_i1	Heat shock protein	Heat shock binding	Stress protein protection
3	TRINITY_DN1844_c0_g1_i1	NAC	NAC transcription factor	Abiotic stress regulation
4	TRINITY_DN19337_c0_g1_i1	WRKY	WRKY transcription factor	Stress signaling
5	TRINITY_DN20358_c0_g1_i1	Aquaporin	Aquaporin PIP2-4-7	Water transport under drought
6	TRINITY_DN20359_c0_g1_i1	Protein kinase	Protein kinase activity	Signal transduction in drought stress
7	TRINITY_DN10994_c0_g1_i1	MYB	MYB transcription factor (partial)	Stress-responsive gene regulation

3.2. Prediction of miRNA-mRNA Interactions

The psRNATarget analysis yielded multiple predicted interactions between conserved plant miRNAs and the reference mRNA sequences of the selected drought-responsive genes. The results, filtered for an expectation score (E) ≤ 4.0, are presented in Table 2. Notably, most interactions were predicted to occur via **cleavage** (20 out of 22 predictions), which is the predominant mode of miRNA action in plants.

Table 2: Predicted miRNAs targeting stress-responsive genes in *Gymnema sylvestre*.

Gene	Predicted miRNA	Expectation	Regulation	Reference Plant
Peroxidase	vvi-miR477b-5p	2.5	Cleavage	Catharanthus roseus

Gene	Predicted miRNA	Expectation	Regulation	Reference Plant
Peroxidase	ath-miR5016	3	Cleavage	Catharanthus roseus
Heat shock protein	osa-miR1438	3	Cleavage	Catharanthus roseus
NAC	gma-miR9742	3.5	Translation	Catharanthus roseus
NAC	ath-miR158b	4	Cleavage	Catharanthus roseus
NAC	gra-miR8751a	4	Cleavage	Catharanthus roseus
NAC	sbi-miR5564b	4	Cleavage	Catharanthus roseus
NAC	zma-miR171c-5p	4	Translation	Catharanthus roseus
MYB	aly-miR838-3p	1.5	Cleavage	Catharanthus roseus
MYB	ath-miR838	3	Translation	Catharanthus roseus
WRKY	bdi-miR7723a-3p	3.5	Cleavage	Catharanthus roseus
WRKY	sly-miR6024	3.5	Cleavage	Catharanthus roseus
WRKY	stu-miR6024-3p	3.5	Cleavage	Catharanthus roseus
Protein kinase	aly-miR399e-5p	3	Cleavage	Catharanthus roseus
Protein kinase	ath-miR5016	3	Cleavage	Catharanthus roseus
Protein kinase	sly-miR6023	3	Cleavage	Catharanthus roseus
Aquaporin	ata-miR2118c-3p	3	Cleavage	Arabidopsis thaliana
Aquaporin	ata-miR2118d-3p	3	Cleavage	Arabidopsis thaliana
Aquaporin	osa-miR2118d	3	Cleavage	Arabidopsis thaliana

International Journal on Science and Technology
One Day National Seminar on
"Advances in Life Sciences for Diversity, Applications, and Human Welfare"
(ALSDAHW – 2025)
Organized by Department of Microbiology & Department of Biotechnology
M.V.S. Government Arts & Science College (Autonomous), Mahabubnagar-509001
E-ISSN: 2229-7677 ● Website: www.ijsat.org ● Email: editor@ijsat.org

The binding details for these interactions, including the precise location on the target mRNA, mode of inhibition, and key biological insights, are provided in Table 3. For example, the peroxidase gene (KT032115.1) is predicted to be cleaved by vvi-miR477b-5p at positions 454-475, and the MYB transcription factor (MT414974.1) is targeted by aly-miR838-3p with high confidence (E=1.5) at positions 815-835. As detailed in Table 3, the NAC transcription factor was predicted to be targeted by five distinct miRNAs, confirming its status as the top network hub identified in our topology analysis.

Table 3: Predicted miRNA-mRNA Interactions for Drought-Responsive Genes

Target Gene (NCBI Accession)	Predicted miRNA	Expectation	Inhibition	Binding Position	Key Functional Insight
Peroxidase (KT032115.1)	vvi-miR477b-5p	2.5	Cleavage	454-475	Targets antioxidant defense; miR477 family linked to stress-responsive regulation.
	ath-miR5016	3.0	Cleavage	1004-1024	May co-regulate peroxidase and protein kinase (see below).
Heat Shock Protein (L14594.1)	osa-miR1438	3.0	Cleavage	1786-1807	Regulates molecular chaperone; potential role in protein stability under drought.
NAC TF (MG676673.1)	gma-miR9742	3.5	Translation	532-552	**Top Network Hub.** Targeted by 5 miRNAs, indicating multi-layer control.
	ath-miR158b	4.0	Cleavage	198-217	
	gra-miR8751a	4.0	Cleavage	159-182	
	sbi-miR5564b	4.0	Cleavage	686-706	
	zma-miR171c-5p	4.0	Translation	586-606	

MYB TF (MT414974.1)	aly-miR838-3p	**1.5**	Cleavage	815-835	**High-confidence interaction.** Links drought signaling to secondary metabolism.
	ath-miR838	3.0	Translation	815-835	Targets same site via translation inhibition.
WRKY TF (MG676674.1)	bdi-miR7723a-3p	3.5	Cleavage	826-849	Regulates stress-signaling integrator.
	sly-miR6024	3.5	Cleavage	118-139	Family-specific targeting pattern observed.
	stu-miR6024-3p	3.5	Cleavage	118-139	
Protein Kinase (JX872402.1)	aly-miR399e-5p	3.0	Cleavage	802-823	Modulates phosphorylation-dependent signaling cascade.
	ath-miR5016	3.0	Cleavage	284-304	**Potential multi-target regulator** (also targets Peroxidase).
	sly-miR6023	3.0	Cleavage	1954-1975	
Aquaporin (EU700487.1)	ata-miR2118c-3p	3.0	Cleavage	696-717	**Conserved targeting.** All three miR2118 members hit identical site for robust water transport regulation.
	ata-miR2118d-3p	3.0	Cleavage	696-717	

International Journal on Science and Technology
One Day National Seminar on
"Advances in Life Sciences for Diversity, Applications, and Human Welfare"
(ALSDAHW – 2025)
Organized by Department of Microbiology & Department of Biotechnology
M.V.S. Government Arts & Science College (Autonomous), Mahabubnagar-509001
E-ISSN: 2229-7677 • Website: www.ijsat.org • Email: editor@ijsat.org

	osa-miR2118d	3.0	Cleavage	696-717	

3.3. miRNA-mRNA Regulatory Network and Hub Analysis

The predicted interactions were used to construct a directed regulatory network (Figure 2). The network comprises 23 nodes (7 mRNA/Gene nodes and 16 miRNA nodes) and 22 edges (interactions). Visual analysis reveals distinct regulatory patterns: the NAC transcription factor is targeted by the highest number of distinct miRNAs (5), followed by WRKY and Protein Kinase (3 each). In contrast, Peroxidase, Heat Shock Protein, Aquaporin, and MYB are each targeted by 2-3 miRNAs.

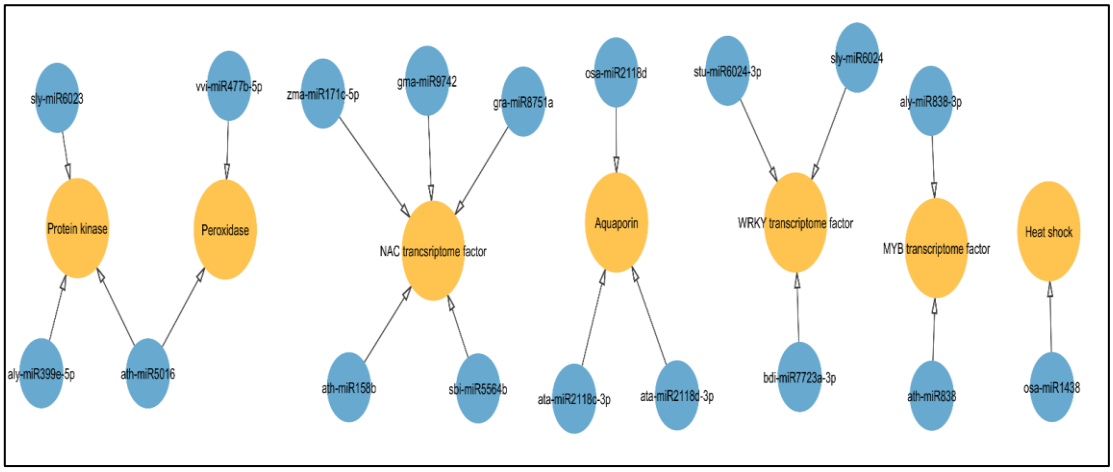

Figure 2. miRNA-mRNA interaction network for drought stress response in *G. sylvestre* (where Blue nodes: miRNAs; Orange nodes: Target mRNA/Genes. Edge direction indicates regulatory action from miRNA to target).

Hub gene analysis using the Degree method in cytoHubba quantitatively confirmed the visual observations. The ranking, shown in Table 4, identifies the NAC transcription factor as the top hub with a degree score of 5, meaning it has connections to five different miRNAs. This highlights its potential role as a central regulatory target in the predicted drought response network.

Table 4: Top 10 hub nodes in the miRNA-mRNA interaction network ranked by Degree centrality.

Rank	Node Name	Score (Degree)	Type
1	NAC	5	Gene
2	Protein Kinase	3	Gene

Rank	Node Name	Score (Degree)	Type
2	Aquaporin	3	Gene
2	WRKY	3	Gene
5	Peroxidase	2	Gene
5	ath-miR5016	2	miRNA
5	MYB	2	Gene
8	aly-miR399e-5p	1	miRNA
8	vvi-miR477b-5p	1	miRNA
8	sly-miR6023	1	miRNA

4. Discussion

4.1. Integration of Predicted Regulatory Network with Established Drought Biology

This in-silico investigation provides the first systematic prediction of miRNA-mediated regulatory networks associated with drought stress responses in the medicinal plant *Gymnema sylvestre*. The core drought-responsive genes identified—Peroxidase, Heat Shock Protein (HSP), NAC, WRKY, Aquaporin, Protein Kinase, and MYB—constitute a functionally coherent module that reflects known components of drought-induced signalling, ROS management, and transcriptional reprogramming in higher plants (Figure 3). The central role of these gene families in stress adaptation has been documented across diverse species (Ferrandino et al., 2023; Hajdarpašić & Ruggenthaler, 2012; Nakashima et al., 2012). Peroxidases act as primary ROS-scavenging enzymes whose activity is tightly integrated into stress signalling, and their regulation by stress-responsive miRNAs has been reported in several crops, linking redox balance to broader metabolic and structural adjustments under abiotic stress (Ferrandino et al., 2023; Choudhury et al., 2017; Singh et al., 2019). In the predicted *G. sylvestre* network, vvi-miR477b-5p and ath-miR5016 are inferred to target peroxidase transcripts, consistent with evidence that grapevine miRNAs, including members of the miR477 family, modulate stress-related transcription factors and secondary metabolic pathways during adverse environmental conditions (Ferrandino et al., 2023; Jiu et al., 2019).

International Journal on Science and Technology
One Day National Seminar on
"Advances in Life Sciences for Diversity, Applications, and Human Welfare"
(ALSDAHW – 2025)
Organized by Department of Microbiology & Department of Biotechnology
M.V.S. Government Arts & Science College (Autonomous), Mahabubnagar-509001
E-ISSN: 2229-7677 • Website: www.ijsat.org • Email: editor@ijsat.org

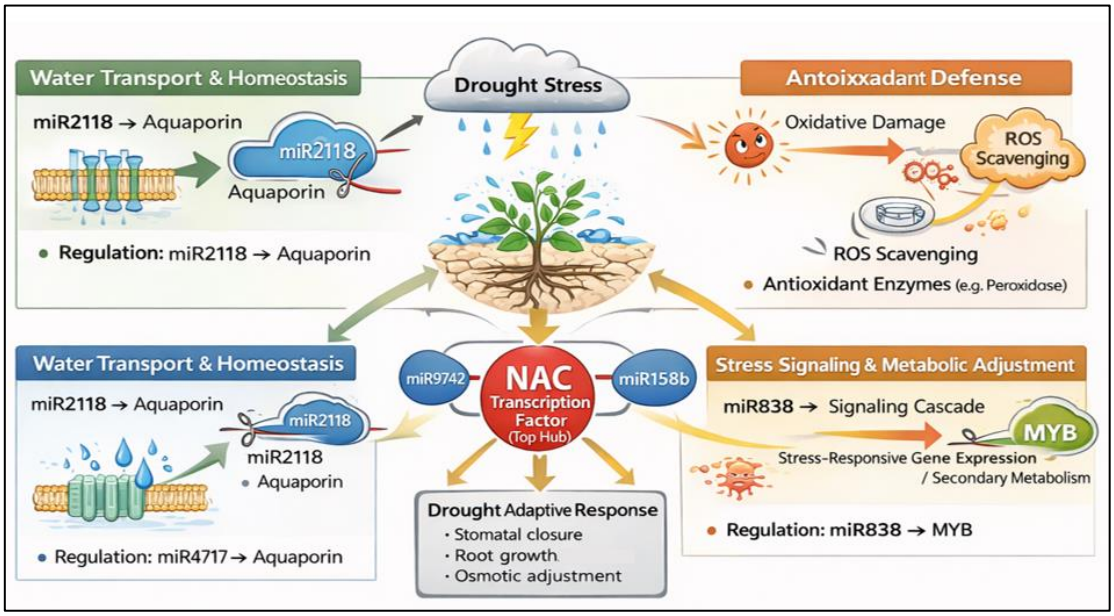

Figure 3. Conceptual model of predicted miRNA-mediated drought regulation in *Gymnema sylvestre* (here miR2118–aquaporin, miR477–peroxidase, and miR838–MYB interacts with the NAC transcription factor, that acts as a central hub, integrating ABA signaling and ROS-responsive pathways).

The high centrality of NAC and WRKY transcription factors within the predicted network underscores their conserved role as master regulators of abiotic stress tolerance. NAC proteins have been widely implicated in the control of drought-adaptive traits such as stomatal behaviour, leaf senescence, and root system architecture, thereby enhancing water conservation and uptake under limited soil moisture (Hajdarpašić & Ruggenthaler, 2012; Nakashima et al., 2012). The finding that a *G. sylvestre* NAC homolog is potentially regulated by five distinct miRNAs suggests a layered regulatory configuration that enables fine-tuning of NAC expression in a spatiotemporal manner, similar to miRNA–NAC modules described in Arabidopsis thaliana under osmotic and drought stress (Hajdarpašić & Ruggenthaler, 2012). Genome-wide analyses in papaya (Carica papaya), a tropical medicinal/fruit species, have identified numerous NAC and WRKY genes that are strongly induced by water deficit, reinforcing the notion that these transcription factor families form central hubs in drought-responsive regulatory networks of non-model plants (Arroyo-Álvarez et al., 2023).

This multi-miRNA targeting of NAC and WRKY homologs in *G. sylvestre* is therefore consistent with miRNA–TF regulatory circuits characterised in Arabidopsis and with NAC/WRKY-centred drought networks reported in tropical species such as papaya (Arroyo-Álvarez et al., 2023; Hajdarpašić & Ruggenthaler, 2012). Likewise, the inclusion of aquaporin and protein kinase genes among the core drought-responsive nodes mirrors stress-induced expression of water transporters and signalling components documented in medicinal plants, where aquaporin up-regulation and kinase-mediated

phosphorylation cascades support rapid osmotic adjustment and downstream transcriptional responses (Singh et al., 2019; Yu et al., 2021). WRKY transcription factors function as key integrators of hormonal and environmental cues, coordinating ROS homeostasis, secondary metabolism, and defence gene activation during abiotic stress exposure (Arroyo-Álvarez et al., 2023; Zhang et al., 2025). Their regulation by stress-responsive miRNAs, including lineage-specific miRNAs in Solanaceae that target WRKY genes, supports a model in which miRNA–TF modules mediate cross-talk between biotic and abiotic stress pathways, thereby enhancing the robustness and flexibility of plant adaptive responses (Zhang et al., 2025). Similar drought-associated modulation of aquaporins and stress-signalling genes in Catharanthus roseus supports the conservation of these core regulatory motifs across medicinal species related to *G. sylvestre*, and lends credence to the broader applicability of the predicted network architecture for understanding drought resilience in phytopharmaceutical crops (Arroyo-Álvarez et al., 2023; Yu et al., 2021).

4.2. Computational Insights and Validation of Predicted miRNA-Gene Modules

The integrated miRNA–mRNA network and target prediction pipeline yielded several high-confidence regulatory modules that align with current knowledge of stress-responsive miRNAs in plants. Among these, the predicted targeting of an MYB transcription factor by aly-miR838-3p (E = 1.5) is particularly notable. In medicinal species such as Salvia miltiorrhiza, miRNAs that target MYB transcription factors have been shown to negatively regulate phenolic acid and tanshinone biosynthesis, thereby linking stress signalling to specialized metabolic pathways (Wu et al., 2021; Zhao et al., 2024). On this basis, the GsMYB–miR838 module predicted in *G. sylvestre* suggests a plausible mechanism by which drought could concurrently influence stress tolerance and the biosynthesis of key medicinal constituents such as gymnemic acids, representing a testable connection between abiotic stress responses and specialized metabolism in this species (Chaudhary et al., 2021; Kim et al., 2025)

Another salient prediction concerns the targeting of Plasma Membrane Intrinsic Protein (PIP) aquaporin genes by members of the miR2118 family. PIP-type aquaporins are central regulators of cellular water flux, and their down-regulation under drought has been associated with reduced water loss and improved maintenance of cell turgor in several species (Surbanovski et al., 2013; Afzal et al., 2016). While miR2118 has classically been characterised as a trigger of phased siRNAs from NBS–LRR resistance gene loci, recent work indicates that stress-responsive miRNAs, including miR2118 family members, can participate in broader regulatory networks extending beyond immune receptors (Chaudhary et al., 2021; Wei et al., 2023). The predicted miR2118–Aquaporin module in *G. sylvestre* therefore points to a previously unrecognised link between miRNA-mediated control of water transport and drought signalling in a medicinal plant, and warrants targeted validation to determine whether this mechanism is conserved across related species.

Network-level hub analysis further highlighted the NAC transcription factor as the most highly connected node in the predicted drought-responsive module. Centrality-based approaches, such as those implemented in cytoHubba, have repeatedly shown that high-degree hubs often correspond to essential regulators in plant stress networks, exerting disproportionate influence on information flow and system

robustness (Chin et al., 2014; Kumar & Mukhtar, 2023). Within this framework, the observation that a single *G. sylvestre* NAC transcript is potentially regulated by multiple miRNAs, including miR9742 and miR158b, suggests an economical regulatory strategy in which a multi-miRNA gatekeeper layer modulates an extensive downstream transcriptional cascade, consistent with miRNA–NAC modules reported in Arabidopsis and other species under abiotic stress (Hajdarpašić & Ruggenthaler, 2012; Nakashima et al., 2012). The GsNAC–miR9742/miR158b interactions therefore emerge as high-priority candidates for experimental confirmation, for example through dual-luciferase reporter assays, degradome analysis, or co-expression profiling in drought-stressed *G. sylvestre* tissues.

4.3. Methodological Strengths, Limitations, and Future Directions

This study's main strength lies in applying an established, plant-focused bioinformatics workflow to a data-poor yet economically important medicinal species. By leveraging sequence homology and functional annotation from model plants and related taxa, it was possible to partially bypass the current lack of a *G. sylvestre* reference genome and to generate concrete, testable regulatory hypotheses. The use of psRNATarget with stringent scoring and target-site accessibility criteria, followed by network-topology-based hub analysis in Cytoscape, adds robustness to the identification and prioritization of candidate miRNA–gene interactions.

Nevertheless, several limitations inherent to in silico prediction must be acknowledged. First, reliance on homologous reference sequences assumes conservation of miRNA binding sites and regulatory logic between *G. sylvestre* and the donor species, which may not always be the case, especially for lineage-specific adaptations. Second, target prediction integrates complementarity and site accessibility but does not capture cellular context, particularly the co-expression of miRNA and target in the same tissues or stress conditions, which is essential for a functional interaction. Third, the current analysis focuses exclusively on conserved miRNAs; species-specific or novel *G. sylvestre* miRNAs, which may be critical for fine-tuned drought adaptation, remain unexplored until small-RNA sequencing data become available

In this context, the computational results should be viewed as a roadmap for experimental work rather than as definitive evidence. Logical next steps include:

1. **Expression profiling:** Performing integrated miRNA and mRNA sequencing on *G. sylvestre* plants exposed to controlled drought stress to confirm differential expression and inverse correlations for key predicted pairs (e.g., miR838–MYB, miR2118–Aquaporin, and miRNA–NAC modules).
2. **Degradome analysis:** Applying degradome-based approaches such as PARE or GMUCT to empirically verify miRNA-directed cleavage at the predicted target sites on *G. sylvestre* transcripts, thereby distinguishing functional targets from false positives.
3. **Functional characterization:** Using transgenic or genome-editing approaches (overexpression, knockdown, or CRISPR-based perturbation) in tractable model systems and, where feasible, in *G. sylvestre* itself to assess the impact of prioritized hub genes (e.g., GsNAC) and their regulatory miRNAs on drought tolerance and associated physiological traits.

5. Conclusion

This work employed a comprehensive in silico strategy to propose a miRNA-mediated regulatory framework for drought stress responses in the medicinal plant *Gymnema sylvestre*. The analysis identified seven core drought-responsive genes and predicted their regulation by 16 conserved miRNAs, primarily through transcript cleavage, thereby outlining a coherent drought-associated module encompassing ROS detoxification, transcriptional control, water transport, and signalling components. Network construction and hub analysis singled out the NAC transcription factor as a central node, supporting its pivotal role in coordinating downstream stress-responsive transcriptional programmes. High-confidence predictions, including the regulation of MYB by aly-miR838-3p and of Aquaporin (PIP) genes by miR2118 family members, suggest mechanistic links between miRNA signalling, secondary metabolism, and water-relation processes in *G. sylvestre*. Although the proposed miRNA–target interactions are still hypothetical and await experimental confirmation, the inferred network offers a clear basis for ranking candidate miRNA–gene pairs and hub regulators for follow-up analyses such as expression profiling, degradome sequencing, and targeted genetic manipulation. Unraveling this regulatory architecture will be crucial for designing molecular interventions to improve drought tolerance in *G. sylvestre*, thereby underpinning its reliable cultivation and phytopharmaceutical exploitation under increasingly unstable climatic conditions

6. References

1. Afzal, Z., Howton, T. C., Sun, Y., & Mukhtar, M. S. (2016). The Roles of Aquaporins in Plant Stress Responses. Journal of developmental biology, 4(1), 9. https://doi.org/10.3390/jdb4010009
2. Arroyo-Álvarez, E., Chan-León, A., Girón-Ramírez, A., Fuentes, G., Estrella-Maldonado, H., & Santamaría, J. M. (2023). Genome-Wide Analysis of WRKY and NAC Transcription Factors in Carica papaya L. and Their Possible Role in the Loss of Drought Tolerance by Recent Cultivars through the Domestication of Their Wild Ancestors. Plants (Basel, Switzerland), 12(15), 2775. https://doi.org/10.3390/plants12152775
3. Ayachit, G., Shaikh, I., Sharma, P., et al. (2019). De novo transcriptome of *Gymnema sylvestre* identified putative lncRNA and genes regulating terpenoid biosynthesis pathway. Scientific Reports, 9, 14876. https://doi.org/10.1038/s41598-019-51355-x
4. Chaudhary, S., Grover, A., & Sharma, P. C. (2021). MicroRNAs: Potential Targets for Developing Stress-Tolerant Crops. Life (Basel, Switzerland), 11(4), 289. https://doi.org/10.3390/life11040289
5. Chin, C. H., Chen, S. H., Wu, H. H., Ho, C. W., Ko, M. T., & Lin, C. Y. (2014). cytoHubba: identifying hub objects and sub-networks from complex interactome. BMC systems biology, 8 Suppl 4(Suppl 4), S11. https://doi.org/10.1186/1752-0509-8-S4-S11
6. Choudhury, F. K., Rivero, R. M., Blumwald, E., & Mittler, R. (2017). Reactive oxygen species, abiotic stress and stress combination. The Plant journal : for cell and molecular biology, 90(5), 856–867. https://doi.org/10.1111/tpj.13299

7. Dai, X., Zhuang, Z., & Zhao, P. X. (2018). psRNATarget: A plant small RNA target analysis server (2017 release). Nucleic Acids Research, 46(W1), W49–W54. https://doi.org/10.1093/nar/gky316
8. Ferrandino, A., Pagliarani, C., & Pérez-Álvarez, E. P. (2023). Secondary metabolites in grapevine: crosstalk of transcriptional, metabolic and hormonal signals controlling stress defence responses in berries and vegetative organs. Frontiers in plant science, 14, 1124298. https://doi.org/10.3389/fpls.2023.1124298
9. Hajdarpašić, A., & Ruggenthaler, P. (2012). Analysis of miRNA expression under stress in Arabidopsis thaliana. Bosnian journal of basic medical sciences, 12(3), 169–176. https://doi.org/10.17305/bjbms.2012.2471
10. Ji, J., Zeng, Y., Zhang, S., Chen, F., Hou, X., & Li, Q. (2023). The miR169b/NFYA1 module from the halophyte Halostachys caspica endows salt and drought tolerance in Arabidopsis through multi-pathways. Frontiers in Plant Science, 13, 1026421. https://doi.org/10.3389/fpls.2022.1026421
11. Jiu, S., Leng, X., Haider, M. S., Dong, T., Guan, L., Xie, Z., Li, X., Shangguan, L., & Fang, J. (2019). Identification of copper (Cu) stress-responsive grapevine microRNAs and their target genes by high-throughput sequencing. Royal Society open science, 6(1), 180735. https://doi.org/10.1098/rsos.180735
12. Kalariya, K. A., Mevada, R. R., & Das, M. (2024). Characterization of phenylalanine ammonia lyase and revealing flavonoid biosynthesis in *Gymnema sylvestre* R. Br through transcriptomic approach. Journal of Genetic Engineering and Biotechnology, 22(1), 100344. https://doi.org/10.1016/j.jgeb.2023.100344
13. Karimipour, R., Koolivand, D., Ghorbani, A., Naderpour, M., & Rostami, M. (2025). Integrated gene network analysis and experimental validation identify key hub genes in potato response to Potato virus Y infection. PLoS ONE, 20(8), e0329747. https://doi.org/10.1371/journal.pone.0329747
14. Kim, J., Dang, Q. H., Kim, S. H., Jeong, D. H., & Suh, M. C. (2025). The miRNA838–KCS2 module is involved in the fine regulation of Arabidopsis wax biosynthesis under water-deficient conditions. Plant Physiology and Biochemistry, 230, 110794. https://doi.org/10.1016/j.plaphy.2025.110794
15. Kumar, N., & Mukhtar, M. S. (2023). Ranking Plant Network Nodes Based on Their Centrality Measures. Entropy (Basel, Switzerland), 25(4), 676. https://doi.org/10.3390/e25040676
16. Luo, Y., Jiao, W., Huang, K., Li, X., Li, J., Wang, M., Zhang, R., & Cao, X. (2025). Comprehensive analysis of sorghum CNGC genes reveals their potential roles in abiotic stress responses. Genes, 16(12), 1405. https://doi.org/10.3390/genes16121405
17. Mukherjee, A., Dwivedi, S., Bhagavatula, L., & Datta, S. (2023). Integration of light and ABA signaling pathways to combat drought stress in plants. Plant Cell Reports, 42(5), 829–841. https://doi.org/10.1007/s00299-023-02999-7
18. Nakashima, K., Takasaki, H., Mizoi, J., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2012). NAC transcription factors in plant abiotic stress responses. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms, 1819*(2), 97–103. https://doi.org/10.1016/j.bbagrm.2011.10.005

19. Preetha, R. A., Davis, G. D. J., Neelima, M. G., Chaitra, B. S., & Kirankumar, B. (2025). Anti-lipidemic and anti-diabetic properties of *Gymnema sylvestre* saponins: An in silico approach. In Silico Pharmacology, 13(2), 94. https://doi.org/10.1007/s40203-025-00369-7
20. Qiu, J., McGaughey, S. A., Byrt, C. S., & Tyerman, S. D. (2025). Post-translational modification acts as a digital-like switch influencing AtPIP2;1 water and cation permeability. Scientific Reports, 15(1), 22552. https://doi.org/10.1038/s41598-025-06200-9
21. Raza, A., Charagh, S., Karikari, B., Sharif, R., Yadav, V., Mubarik, M. S., Habib, M., Zhuang, Y., Zhang, C., Chen, H., Varshney, R. K., & Zhuang, W. (2023). miRNAs for crop improvement. Plant physiology and biochemistry : PPB, 201, 107857. https://doi.org/10.1016/j.plaphy.2023.107857
22. Razalli, I. I., Abdullah-Zawawi, M. R., Zainal Abidin, R. A., Harun, S., Che Othman, M. H., Ismail, I., & Zainal, Z. (2025). Identification and validation of hub genes associated with biotic and abiotic stresses by modular gene co-expression analysis in Oryza sativa L. Scientific reports, 15(1), 8465. https://doi.org/10.1038/s41598-025-92942-5
23. Singh, A., Kumar, A., Yadav, S., & Singh, I. K. (2019). Reactive oxygen species-mediated signaling during abiotic stress. Plant Gene, 18, 100173. https://doi.org/10.1016/J.PLGENE.2019.100173
24. Šurbanovski, N., Sargent, D. J., Else, M. A., Simpson, D. W., Zhang, H., & Grant, O. M. (2013). Expression of Fragaria vesca PIP aquaporins in response to drought stress: PIP down-regulation correlates with the decline in substrate moisture content. PloS one, 8(9), e74945. https://doi.org/10.1371/journal.pone.0074945
25. Suzuki, T., Ikeda, S., Kasai, A., Taneda, A., Fujibayashi, M., Sugawara, K., Okuta, M., Maeda, H., & Sano, T. (2019). RNAi-mediated down-regulation of Dicer-like 2 and 4 changes the response of 'Moneymaker' tomato to potato spindle tuber viroid infection from tolerance to lethal systemic necrosis, accompanied by up-regulation of miR398, 398a-3p and production of excessive amounts of reactive oxygen species. Viruses, 11(4), 344. https://doi.org/10.3390/v11040344
26. Wang, Y., Ruan, Q., Qiu, X., Wang, X., Chen, L., Liu, H., Wang, T., Zhang, D., Wang, B., Zhao, Y., & Wei, X. (2025). miR164-targets involved in plant biological processes and stress response: A review. Plant Physiology and Biochemistry, 229(Pt B), 110508. https://doi.org/10.1016/j.plaphy.2025.110508
27. Wei, L., Du, Y., Xiang, J., Zheng, T., Cheng, J., & Wu, J. (2023). Integrated mRNA and miRNA transcriptome analysis of grape in responses to salt stress. Frontiers in plant science, 14, 1173857. https://doi.org/10.3389/fpls.2023.1173857
28. Wu, S., Zhu, B., Qin, L., Rahman, K., Zhang, L., & Han, T. (2021). Transcription Factor: A Powerful Tool to Regulate Biosynthesis of Active Ingredients in Salvia miltiorrhiza. Frontiers in plant science, 12, 622011. https://doi.org/10.3389/fpls.2021.622011
29. Yan, W., Fu, R., Huang, X., & Zhan, A. (2025). Dynamic and functional miRNA-mediated gene regulations in response to recurrent environmental challenges during biological invasions. Molecular Ecology, 34(20), e17749. https://doi.org/10.1111/mec.17749

30. Yin, Z., Li, Y., Zhu, W., Fu, X., Han, X., Wang, J., Lin, H., & Ye, W. (2018). Identification, characterization, and expression patterns of TCP genes and microRNA319 in cotton. International Journal of Molecular Sciences, 19(11), 3655. https://doi.org/10.3390/ijms19113655
31. Yu, B., Pan, Y., Liu, Y., Chen, Q., Guo, X., & Tang, Z. (2021). A comprehensive analysis of transcriptome and phenolic compound profiles suggests the role of flavonoids in cotyledon greening in Catharanthus roseus seedling. Plant physiology and biochemistry : PPB, 167, 185–197. https://doi.org/10.1016/j.plaphy.2021.07.028
32. Yue, Z., Zhang, G., Ercisli, S., Wang, J., Wang, J., Li, J., Chen, T., & Liu, Z. (2025). Identification and functional characterization of MYB genes regulating polyphenol biosynthesis in cabbage for resistance to Xanthomonas campestris pv. campestris. Plant Physiology and Biochemistry, 222, 109714. https://doi.org/10.1016/j.plaphy.2025.109714
33. Zhang, X., Liu, W., Yin, Y., Zheng, J., Li, J., Tan, X., & Wu, L. (2025). WRKY transcription factors participate in abiotic stress responses mediated by sugar metabolism. Frontiers in plant science, 16, 1646357. https://doi.org/10.3389/fpls.2025.1646357
34. Zhou, H., Jiang, M., Li, J., Xu, Y., Li, C., & Lu, S. (2024). Genome-Wide Identification and Functional Analysis of Salvia miltiorrhiza MicroRNAs Reveal the Negative Regulatory Role of Smi-miR159a in Phenolic Acid Biosynthesis. International journal of molecular sciences, 25(10), 5148. https://doi.org/10.3390/ijms25105148