Structural characterisation of stress-responsive miRNA precursors of rice (Oryza sativa L.).

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Research Article

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Abstract

MicroRNAs (miRNAs) play crucial roles in regulating metabolic processes and orchestrating responses to countless environmental stresses and pathogen invasion. These regulatory functions are largely governed by the secondary structural conformation of the miRNA precursor molecules. Rice (*Oryza sativa* L.) the most important staple food, is constantly challenged by diverse biotic and abiotic stress factors that limit its productivity. However, the knowledge of the structural diversity of stress-responsive precursor miRNAs (pre-miRNAs) of rice remains inadequate. In this study, nearly150stress-responsive pre-miRNAs of rice were retrieved from miRBase and individually analysed for their secondary structures using the UNAFold server. Four distinct structural patterns were identified, primarily consisting of stems, internal loops, bulges, and terminal loops. Structural analysis revealed majority of precursor with 1–2 stems and 1–2 terminal loops. However, internal loops and bulges showed greater variability in number. Approximately, 34% and 23% of the precursors completely lacked internal loops and bulges respectively. Free energy (ΔG) analysis indicated variations in structural stability, with osa-miR7695 being the most stable (–342.00 kcal/mol) and osa-miR395 the least stable (–28.70 kcal/mol). Furthermore, the strong concordance between secondary structure clustering (heat map) and subsequent Principal Component Analysis (PCA) suggests that the variations in the secondary structure of the precursor may influence its biogenesis and function. Collectively, these results provide valuable insights on structural information for designing miRNA-based strategies to enhance stress tolerance in rice.

Introduction

Rice (*Oryza sativa* L.), a monocotyledonous plant belonging to the family Poaceae, is the major dietary staple for over half of the world's population. It serves as a central component of daily nutrition, especially in Asia, Africa, and Latin America. Rice production is constantly being threatened by several biotic and abiotic stress factors. Abiotic stresses, such as drought, salinity, submergence, extreme temperatures, and nutrient deficiency significantly impact crop yield and quality (Radha et al.2023). For example, drought stress reduces water availability, inhibiting photosynthesis and limiting nutrient uptake, which severely hampers growth and productivity (Pandey and Shukla 2015). Salinity stress disrupts ionic balance and osmotic pressure, causing chlorosis and reduced biomass (Munns and Tester 2008). Biotic stresses, including bacterial blight, blast disease, and pest attacks, further threaten rice health and yield (Skamnioti and Gurr 2009). The three most adverse rice diseases are sheath blight, bacterial blight (BB), and rice blast. These diseases are mediated by the necrotrophic fungus *Rhizoctonia solani (R. solani*), the hemi biotrophic Gram-negative bacterium *Xanthomonas oryzae*pv. *oryzae* (Xoo), and the hemi biotrophic fungus *Magnaporthe oryzae* (Lee and F. N1983).

In rice, microRNAs (miRNAs) are essential for controlling gene expression during numerous stress reactions such as pathogen assaults, drought, salinity, and cold, by modifying the expression of stress-responsive genes at the post-transcriptional level. These genes preserve cellular homeostasis and increase stress tolerance (Li et al. 2014) in rice. For instance, osa-miR169 was confirmed as the only member induced by drought among the osa-miR-169 family. osa-miR-169g is regulated by dehydration-

responsive elements (DREs) in its promoter region, which are likely to be activated by CBF/DREB transcription factors under drought conditions (Zhao et al. 2007). The miRNA osa-miR393 is upregulated and targets auxin receptors TIR1, AFB2, AFB3 (Xia et al. 2012) while osa-miR414, osa-miR164 and osa-miR408 are down-regulated (Umate 2010) when induced by salinity stress. To date, osa-miR398 showed increased expression in response to oxidative stress and targets copper SOD enzyme through transcriptional regulation, nutrient transport, auxin homeostasis, cell proliferation, and programmed cell death (Li et al. 2011).It was reported thatosa-miR160 and osa-miR167 are key regulators of auxin signalling in rice, primarily through their targeting of the transcription factors such as auxin response factors (ARFs) (Meng et al. 2010). Again, osa-miR528 showed upregulation in response to Cd (heavy metal) stress whereasosa-miR168 is down regulated under heavy metal stress condition (Ding et al. 2011). Similarly, multiple miRNAs in rice are responsible for several biotic stresses experienced during rice blast (Jia et al. 2025), or blight diseases. The osa-miR7695 targets gene OsNramp6, an iron transporter and enhances ROS. It limits pathogen growth by metal sequestration and oxidative burst (Quoc et al. 2019; Sánchez-Sanuy et al. 2019). The miRNA osa-miR160a targets OsARF8 and leads to increased ROS production, callose accumulation, and the expression of defence-related genes, thereby enhancing rice immunity (Wang et al. 2018).Likewise, osa-miR398b is reported to boost resistance through increased ROS production (Li et al. 2019).The osa-miR164 negatively regulates immunity to *M. oryzae* and its overexpression increases susceptibility by suppressing defence genes and H_2O_2 production. Mutation of its target, OsNAC60, leads to susceptibility, while its overexpression induces cell death (Wang et al. 2018). osa-miR169o negatively regulates rice immunity against *Xanthomonas oryzae*pv. *oryzae* (Xoo) by suppressing the expression of NF-YA transcription factors, particularly NF-YA4. Overexpression of osa-miR169o leads to reduced transcription of NF-YA4 and other NF-YA genes, which in turn downregulates pathogenesis-related (PR) genes. This suppression impairs the plant's innate immune response, resulting in increased susceptibility to Xoo infection (Yu et al. 2018). osa-miR395a negatively regulates *R. solani* infection in rice by targeting and cleaving mRNAs encoding pentatricopeptide repeat (PPR) proteins, which are involved in chloroplast RNA processing. This regulation may contribute to chloroplast degradation, a defence response limiting pathogen spread (Srikakulam et al. 2022). Through the regulation of target genes, miRNAs coordinate hormonal signalling, ROS dynamics, and defence pathways, thereby maintaining cellular homeostasis. Figure 1 represents various stress-responsive and regulatory microRNAs involved in abiotic and biotic stresses in rice.

Since the biological activity of miRNAs is strongly influenced by the secondary structure of their precursor molecules, the structural features become critical determinants of target recognition and gene regulation. These features also affect precursor processing, miRNA maturation, stability, and abundance. In addition, variations in the structural elements such as loops, bulges and stem structure of pre-miRNAs play a critical role in precursor stability, accumulation and Dicer processing efficiency, which may ultimately influence the gene regulation. Despite the increasing knowledge of stress-responsive miRNAs and their target genes, little is known about the contributions of their secondary structures to miRNA biogenesis and regulatory functions.

Hence, the present study aims to provide insights into the secondary structural characteristics of predominantly stress-responsive rice pre-miRNAs. This study may support the potential applications of miRNAs in developing rice varieties with enhanced stress tolerance and improved crop resilience.

Materials and methods

miRNA sequence Library construction

The miRBase database is a searchable database of published miRNA sequences and annotation. Each entry in the miRBase Sequence database represents a predicted hairpin portion of a miRNA transcript (termed mir in the database), with information on the location and sequence of the mature miRNA sequence (termed MIR). Stress-responsive miRNA (as previously reported in journals) precursor sequences (approximately 150 precursors) of rice (osa-mir) were downloaded from the miRBase database release version V22 (https://mirbase.org/)

Sequence submission for secondary structure generation

Each miRNA precursor sequence was submitted to the UNAFold server (https://www.unafold.org/mfold/applications/rna-folding-form.php) to predict its secondary structure. For all the precursors, potentially stable secondary structures were generated by UNAFold algorithm. The parameters were used for the secondary structure prediction using UNAFold - minimum free energy and partition function; dangling energy on both sides of the helix in any case; rescale energy parameters at a given temperature 37°C; interactive RNA secondary structure plot; RNA secondary structure plots with reliability annotation (Sengupta et al 2025). Finally, these structures of precursors were manually curated using rules of Zhang et al. 2005.

Heat map generation using R studio software.

A heat map of the two-way hierarchical clustering of miRNA secondary structure data of all three species was obtained from the FAIR server. Cluster analysis classified the samples into groups based on the number of stems, bulges, internal loops, and terminal loops. Red colour represents the highest number and blue colour represents the lowest (absence). The Heat map was generated using R Studio (https://posit.co/download/rstudio-desktop/).

PCA Graph generation

PCA graph was generated by RStudio (version 2026.07.0.) [https://www.rdocumentation.org/packages/pcaMethods/versions/1.64.0]. Essentially it was used for dimensionality reduction and separating the distribution into two major principal components with PC1 capturing the largest variance followed by PC2. In this analysis PCA was used for identifying the

variances among the identified structural components to understand which variable(s) had significant impact on the secondary structure stability.

Results

Secondary Structure analysis

The analysis of stem regions, which form the double-helical portions of the secondary structure through Watson-Crick and wobble base pairing, revealed considerable variation across the dataset (Bansal2003). The number of stems ranged from 1 to 6, with approximately 80% of precursors containing 1–2 stems. The precursor osa-miR399d exhibited the maximum stem count of 6, while the majority of sequences displayed the canonical single-stem hairpin structure typical of plant miRNA precursors. Internal loop analysis revealed substantial structural complexity, with counts ranging from 0 to 6 across different precursors. Notably, osa-miR408 and osa-miR167e displayed the highest internal loop complexity with 6 loops each, while 34% of the precursors contained no internal loops. osa-miR156b exhibited the maximum bulge count of 7, while 23% of precursors contained no bulges (**Supplementary Table 1**)

Thermodynamic Stability Analysis

The thermodynamic analysis revealed a broad range of free energy values spanning from − 28.70 to -342.00 kcal/mol, indicating substantial diversity in structural stability among rice miRNA precursors. The most thermodynamically stable structure was observed in osa-miR7695(ΔG = -342.00 kcal/mol), while osa-miR395u exhibited the least stable configuration (ΔG = -28.70 kcal/mol). This reflects approximately 12-fold difference in stability among different miRNA families.

Principal Component Analyses and Clustering:

To understand the overall variation among the three structural features of the miRNA sequences under study, a Principal Component Analysis (PCA) was performed. Results indicated that the total variance was sufficiently explained by the two principal components consisting of 65.2% (PC1) and 34.8% (PC2) of the total variance **(Supplementary File 2)** (Fig. 3). Bulges and stem loops were separated under PC1 while PC2 captured the other structural differences. The distinct separation of the three structural groups along the principal component axes indicates substantial differences in their miRNA-associated features. The complete variance explained by the first two components and the absence of overlap among the groups suggest that Bulges, Stem, and Loop regions exhibit unique structural and functional characteristics that may contribute differentially to miRNA biogenesis and regulatory activity. Hierarchical clustering and heatmap analysis presented the distribution patterns of rice miRNAs across Bulges, Stem, and Loop regions (Fig. 2). The heatmap provided clarity on the structural categories and their associations with different precursor sequences with region specific enrichment serving as one important axis of difference. Unsupervised clustering showed that Stem and Loop regions were more closely related to each other than to Bulges, indicating similar miRNA distribution profiles. We found that

several miRNA families formed distinct clusters for example - several osa-miR156, osa-miR166, osa-miR169, osa-miR395, osa-miR399, and osa-miR171 family members, frequently occur within related clusters, suggesting shared structural preferences or coordinated regulatory functions. Table 1 highlights the structural similarities or dissimilarities among the major miRNA families that have been reported to be stress associated in rice. The results further amplify the fact that secondary structural features might be specific for a particular family of miRNA. And suggest that miRNA occurrence is strongly influenced by precursor secondary structure and that different structural elements may contribute uniquely to miRNA processing, stability, and regulatory function.

Table 1

Average secondary structural features of major stress-associated miRNA families in rice. The table highlights the structural similarities and dissimilarities among representative pre-miRNAs, including the mean number of stems, internal loops, bulges, terminal loops, and minimum free energy (ΔG), providing an overview of the structural diversity within stress-responsive rice miRNA families.

SL No.	miRNA Family	Associated Stress	Average stems	Average Terminal Loops	Average Bulges	Range of Free Energy ΔG ranges
1	osa-miR160a	Regulates auxin signalling through ARFs, Enhances resistance against *Magnaporthe oryzae*, *Xoo*, and *R. solani*	1	1	1	−49.10 kcal/mol
2	osa-miR156	Downregulated under Cd stress, increases susceptibility to blast and bacterial blight by suppressing SPL genes	2	2	3	−57.80 kcal/mol to − 102.10 kcal/mol
3	osa-miR159	Upregulated during drought, enhances blast resistance by suppressing GAMYB genes; improves *Xoo* resistance	3	1	3	-78.10 kcal/mol to -112.80 kcal/mol
4	osa-miR164a	Downregulated under salt stress, negatively regulates immunity against blast and bacterial blight	4	1	4	-68.30 kcal/mol
5	osa-miR166h/k	Downregulated under Cd stress, positively regulates blast resistance via ethylene signalling	3	2	1	-65.70 kcal/mol to -69.40 kcal/mol
6	osa-miR167d	Regulates auxin signalling, negatively regulates blast and bacterial blight resistance	3	1	1	-52.30 kcal/mol
7	osa-miR168	Downregulated under Cd stress, negatively regulates blast resistance	3	2	2	-37.00 kcal/mol to -52.20 kcal/mol
8	osa-miR169	Upregulated during drought and oxidative stress, negatively regulates blast and	3	2	2	−40.20 kcal/mol to − 113.10 kcal/mol

SL No.	miRNA Family	Associated Stress	Average stems	Average Terminal Loops	Average Bulges	Range of Free Energy ΔG ranges
		bacterial blight resistance				
9	osa-miR171b	Upregulated during drought, Enhances JA-mediated immunity against blast	3	2	0	-54.40 kcal/mol
10	osa-miR395	Upregulated during drought, Positive regulator against Xoo; involved in defence against *R. solani*	2	2	3	−28.70 kcal/mol to − 69.20 kcal/mol
11	osa-miR398b	Upregulated during oxidative stress, Enhances blast resistance through ROS accumulation	2	0	0	-62.20 kcal/mol
12	osa-miR319	Upregulated during drought, negatively regulates blast resistance	3	1	1	-98.80 kcal/mol to -101.50 kcal/mol
13	osa-miR393	Upregulated during drought and salt stress, Associated with auxin-mediated stress signalling and defence pathways	3	2	2	-58.50 kcal/mol to -61.60 kcal/mol
14	osa-miR162	Downregulated under Cd stress, positively regulates blast resistance	3	2	2	-72.70 kcal/mol
15	osa-miR1425	Upregulated during oxidative stress, Potential defence-related role through PPR proteins	3	1	0	-46.00 kcal/mol
16	osa-miR397	Upregulated during oxidative stress	4	1	0	-50.60 kcal/mol to -58.20 kcal/mol
17	osa-miR408	Downregulated during salt stress	6	3	1	-89.10 kcal/mol

Discussion

The presence of internal loop is functionally significant. This is because internal loops contribute to processing efficiency. Dicer tends to produce longer miRNAs from precursor arms with higher numbers of unpaired nucleotides, suggesting a possible direct influence of internal loop configuration on mature miRNA length and also on target recognition (Danaee et al. 2018). The analysis of the secondary structure showed the presence of 1 to 4 terminal loops, 85% of the precursors having 1–2 terminal loops. The terminal loop is a key structural component that ends the stem-loop structure and acts as a processing signal to the miRNA machinery. The most abundant precursors contained a single terminal loop, in agreement with the canonical hairpin structure. Bulge analysis had the greatest structural variation, with counts ranging from 0 to 7. Bulges are areas of structural asymmetry that can have an impact on thermodynamic stability and processing accuracy. Bulges may influence the precision of Dicer cleavage as these structural features modify the relative position of the enzyme to the cleavage sites (Hutvágner and Zamore 2002).

In addition, the range of minimum free energy (MFE) gives an idea about the stability of the secondary structure of pre-miRNA. The secondary structure with the lower Gibbs free energy (ΔG) is the most stable structure (Rolle et al. 2016). Lower free energy values correspond to higher thermodynamic stability, which is associated with higher structural complexity in terms of stem length and base-pairing density. Highly stable structures (ΔG − 100.00 kcal/mol) were shown to have better resistance to nuclease degradation and correlate with longer half-lives in cellular environments. These findings concur with the inferences of Belter et al 2014, which indicate that hairpin miRNAs enjoy greater half lives in the cell as compared to free miRNA molecules – the secondary structures conferring nuclease resistance. The average MFE values have been reported for pre-miRNAs from *Arabidopsis* was − 57 kcal/mol (Bonnet et al. 2004; Debat and Ducasse 2014) and − 45 kcal/mol for wheat (Kumar et al. 2014). In comparison, rice miRNAs showed much lower MFE values with greater thermodynamic stability. The more negative values can also be attributable to longer precursor length. Additionally, approximately 12-fold differences in stability of the secondary structures reflect substantial diversity which may indicate differences in functional requirements and processing mechanisms among different miRNA families in rice.

The PCA and heatmap analyses further revealed that Bulges, Stem, and Loop regions represent distinct miRNA signatures. The PCA consistently identifies overall structural differentiation, while the heatmap identifies specific miRNA clusters driving these differences. The concordance between both analyses are consistent with the hypothesis that precursor secondary structure significantly influences miRNA organization and may play an important role in regulating miRNA biogenesis and function in rice.

Conclusion

A comprehensive analysis of the pre miRNAs of rice was performed to identify variations in the number of stems, loops and bulges. Some of these structural patterns were found to be unique to several stress

associated miRNA families and were subjected to multivariate analyses such as PCA and heatmap clustering. Future studies should focus on elucidating the structure function correlation of osa-miRNAs and work toward identifying novel stress-responsive osa-miRNAs in rice. Such investigations will enhance our understanding of miRNA-mediated regulatory networks and facilitate the development of rice varieties with improved stress tolerance and better adaptive potential.

Declarations

Authors' contributions

SM- data curation, investigation, writing—original draft, formal analysis

SG- conceptualization, investigation, writing—review and editing

SSG-conceptualization, investigation, writing—original draft, review and editing

All authors critically proofread the manuscript.

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Data availability

All data generated or analysed during this study are included in this published article and its Supplementary Information.

Statements and Declarations

The authors declare that they have no conflict of interest.

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Figures

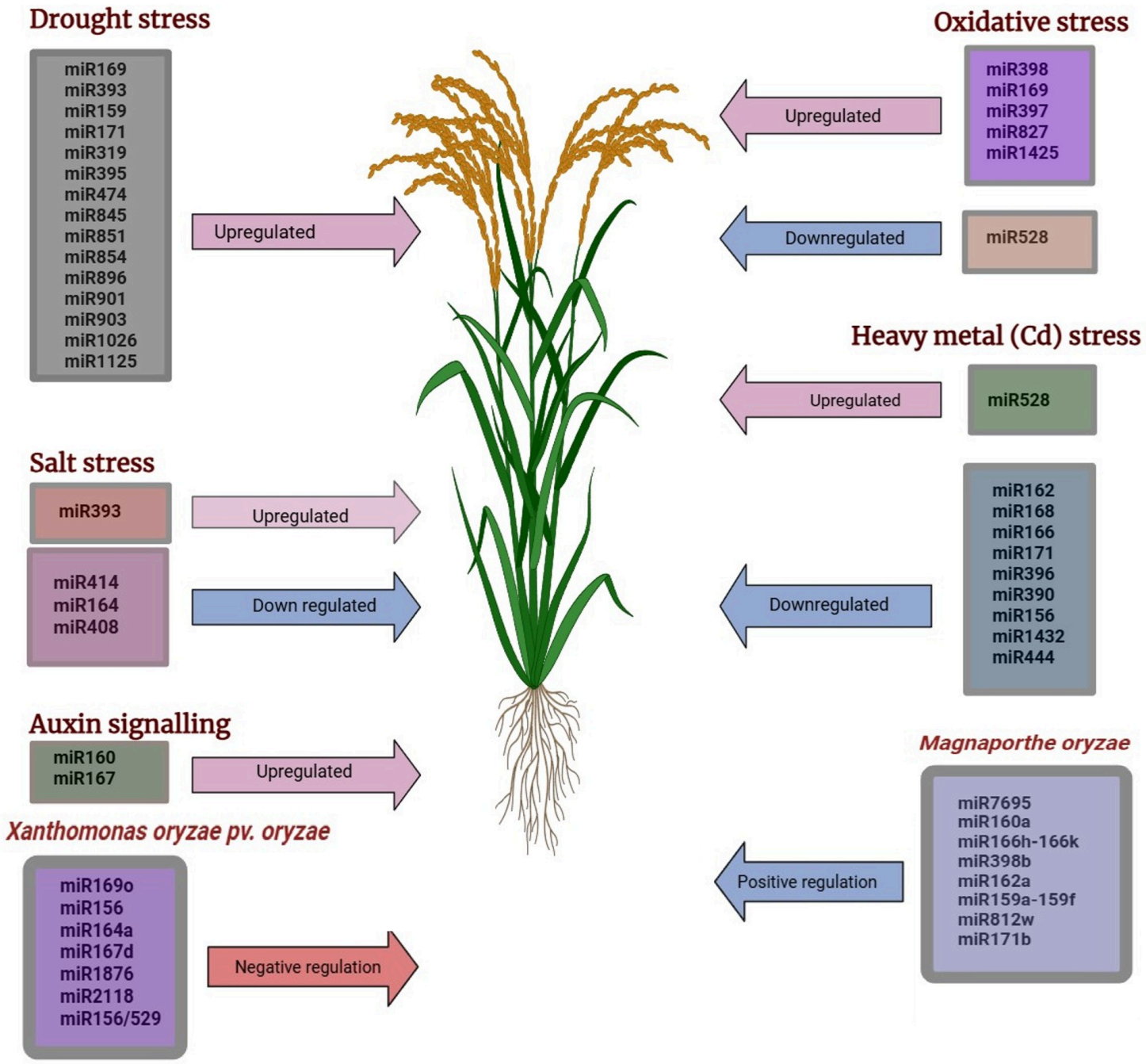

Figure 1

Schematic representation of stress-responsive and regulatory microRNAs involved in various abiotic and biotic stresses in rice. Arrows indicate the direction of regulation (upregulation, downregulation, or positive regulation) under the respective stress or signalling conditions.

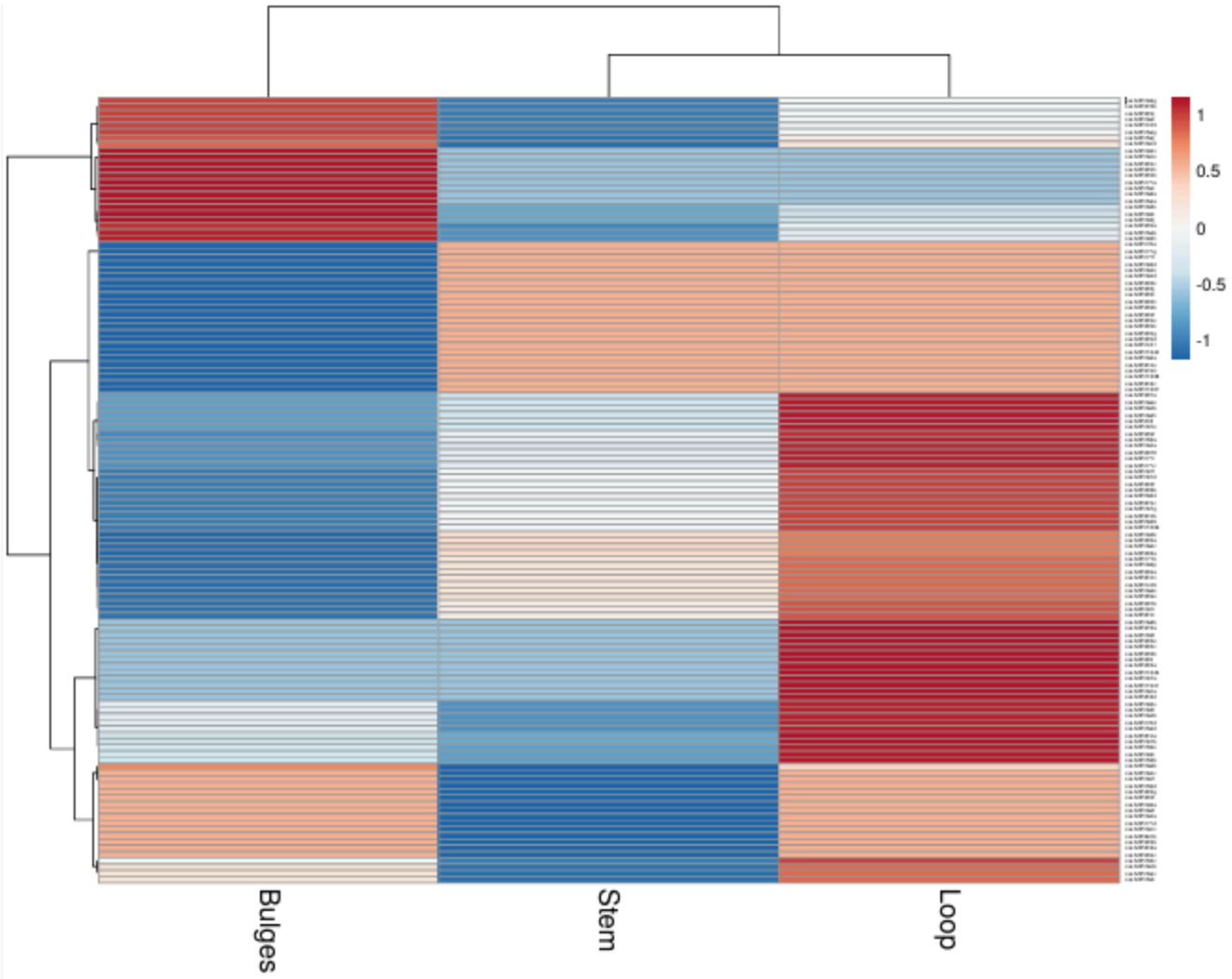

Figure 2

Heat map of two-way hierarchical clustering of microRNA (miRNA) secondary structure data of *Oryza sativa* obtained from Fair server (Sample miRNA sequences are shown at the right. Each row represents one miRNA. The miRNA clusters are represented as branch connections on the left. Cluster analysis classified the samples into groups based on number of stems, bulges, number of internal loops and terminal loops. Red colors represent the highest number and blue represent the lowest). The Heat map was generated using R Studio (https://posit.co/download/rstudio-desktop/).

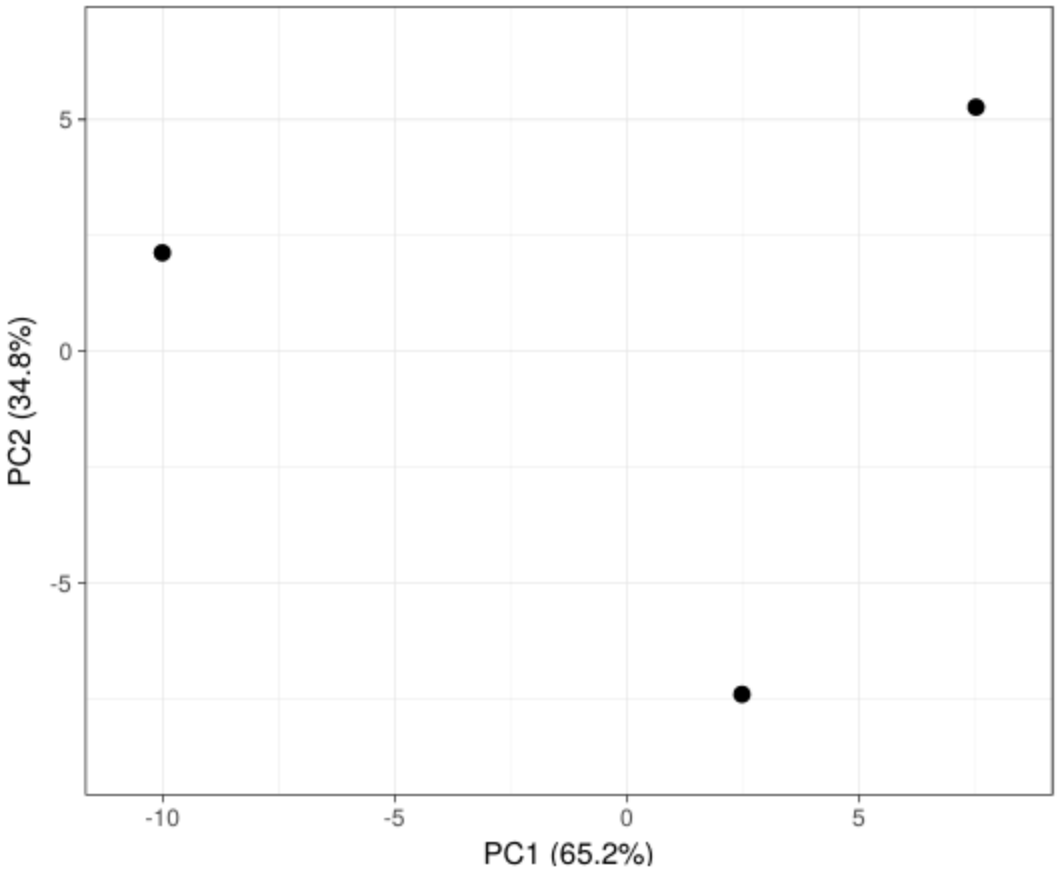

Figure 3

Principal Component Analysis (PCA) for structural features of rice pre-miRNAs. The PCA score plot illustrates the distribution of rice pre-miRNAs based on their structural characteristics. The first principal component (PC1) accounts for 65.2% of the total variance, while the second principal component (PC2) explains 34.8%, together capturing 100% of the observed variation.