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RESEARCH ARTICLE

Comparative analysis of conserved miRNAs in maize germination and vegetative development

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Abstract

Seed germination represents a metabolically active but transcriptionally limited transition that depends largely on post-transcriptional regulation. MicroRNAs (miRNAs) are key regulators of gene expression through mRNA slicing or translational inhibition, and may play an important role in controlling stored mRNAs during early germination. In this study, we combined bioinformatic analysis and experimental validation to identify conserved miRNAs involved in maize (Zea mays) germination. Mature miRNA sequences from Zea mays and Oryza sativa were retrieved from PMRD and miRBase v22.1, identifying conserved candidates across cereals. From this set, ten miRNAs were selected based on sequence conservation and reported roles in seed biology. Target prediction using thermodynamic modelling (ViennaRNA; MFE ≤ −25 kcal/mol) revealed potential interactions between specific miRNAs and mRNAs encoding ribosomal proteins, suggesting regulation of the translational machinery. Expression analysis by Northern blot across germination stages (0, 6, 12, and 24 h) and vegetative tissues showed distinct patterns. miR528 and miR396a were highly expressed in seeds but absent in vegetative tissues, while miR408 decreased after imbibition. miR414 and miR415 displayed transient expression during germination. Notably, miR160a was strongly expressed during germination, suggesting a role beyond vegetative development. These results support a model in which miRNAs regulate stored mRNAs and translation during germination. This work provides candidate miRNAs for functional studies and potential targets for improving seed vigor and early seedling establishment in maize.

Keywords: microRNA; maize; seed germination; ribosomal proteins; Northern blot.

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1. Introduction

Gene expression in plants is regulated at both transcriptional and post-transcriptional levels. Among the latter, microRNAs (miRNAs), which are small, non-coding RNAs typically 19 – 24 nucleotides long, play critical roles by promoting mRNA cleavage or inhibiting translation (Li et al., 2024; Ding & Zhang, 2023; Lanet et al., 2009). While mRNA decay is the predominant mechanism in plants, translational repression via miRNA binding to 5′ or 3′ untranslated regions (UTRs) is increasingly recognized, particularly under developmental or stress-related conditions (Tang et al., 2012; Guo et al., 2022; Zhao et al., 2025). Additionally, 5′ to 3′ mRNA decay has been shown to contribute significantly to the regulation of seed germination and dormancy (Basbouss-Serhal et al., 2017; Gran et al., 2025). Seeds represent a distinct developmental stage marked by dormancy and desiccation tolerance. During maturation, large pools of mRNAs are stored in a transcriptionally silent state, stabilized by RNA-binding proteins (RBPs), P-bodies, and stress granules (Bai et al., 2020). The physiological importance of these stored mRNAs during germination has been reviewed extensively (Sano et al., 2020; Li et al., 2024). Upon imbibition, these mRNAs are selectively translated to drive germination. The role of decapping proteins in miRNA accumulation and stability further highlights the complexity of post-transcriptional control during seed development (Motomura et al., 2012).

A growing body of evidence suggests that ribosomal proteins (RPs) and their mRNAs are not merely passive components of the translational machinery, but active regulatory nodes in developmental and stress-response networks. In plants, RP mRNAs are

among the most abundant transcripts in seeds and are rapidly recruited to polysomes upon imbibition, suggesting their prioritized translation is essential for germination (Bai et al., 2020). Beyond their structural role, RPs can act as signaling molecules: for example, mutations in specific RPs in Arabidopsis lead to developmental defects independent of global translation rates, indicating "ribosome specialization" or "extra ribosomal functions" (Xiong et al., 2021; Martinez-Seidel et al., 2020; Nieves-Cordones et al., 2025). Furthermore, RP mRNAs are enriched targets of miRNAs in multiple plant species, implying a conserved layer of post-transcriptional regulation over the ribosome itself (Li et al., 2013; Zhang et al., 2009).

This leads to our central hypothesis: that conserved miRNAs regulate the seed-to-seedling transition, in part, by targeting ribosomal protein mRNAs, thereby modulating not only translational capacity but also ribosome-mediated signaling pathways critical for germination. This regulatory logic is supported by studies showing that miRNAs like miR169 and miR397 directly target RP transcripts in rice and maize, influencing development and stress responses (Ding et al., 2012; Zhang et al., 2013). The targeting of RP mRNAs by miRNAs may serve as a rapid "dimmer switch" to fine-tune proteome remodeling during the metabolically explosive phase of germination, without requiring new transcription, a mechanism that recent work on translational reprogramming under stress underscores as broadly relevant for crop resilience (Tian et al., 2025).

miRNAs are key regulators of development, influencing processes such as flowering, embryo formation, and seedling establishment (Guo et al., 2022), and classic studies have demonstrated their role in controlling leaf morphogenesis and organ patterning (Palatnik et al., 2003). In maize, miR396 regulates stigma formation, miR167 targets ARF6 and ARF8, miR156 and miR159 control seed size and grain weight via SPL and MYB transcription factors (Tang et al., 2012; Zhang et al., 2013). miR528 also plays a role in somatic embryogenesis, showing stage-specific expression (Luján-Soto et al., 2021). In rice, miRNA-mediated regulation contributes to grain yield. Repression of miR156 enhances panicle branching (Xue et al., 2021), while overexpression of miR397a/b increases grain size by targeting OsLac, a gene involved in brassinosteroid signaling (Zhang et al., 2013; Wang et al., 2023). These findings underscore the agronomic potential of miRNA modulation in monocots. Beyond development, miRNAs mediate hormonal and stress responses during germination. miR160 targets ARF10, influencing ABA sensitivity and meristem function (Dong et al., 2022; Sarkar Das et al., 2018). miR164 and miR169 regulate stress-related transcription factors NAC1 and NFYA5 (Ding et al., 2012), while miR402 and miR159 control germination under dehydration and salt stress via epigenetic and hormonal pathways (Xue et al., 2021; Wang et al., 2023).

This study examines the expression of miRNAs during *Zea mays* germination. Conserved miRNAs from maize and rice were identified, their interactions with ribosomal protein mRNAs predicted using minimum free energy (MFE) calculations, and expression validated by Northern blot. By focusing on RP mRNAs as preferential targets, this work tests the hypothesis that miRNAs orchestrate germination by directly regulating the core translational apparatus and its associated signaling functions. This work contributes to the understanding of miRNA-mediated regulation during the early stages of maize development and proposes several conserved miRNAs as promising targets for functional validation.

2. Methodology

The study followed four sequential stages (Figure 1): (1) retrieval and conservation-based filtering of miRNA sequences from PMRD, miRBase v22.1, and Phytozome, yielding a panel of 10 conserved miRNAs shared between *Zea mays* and *Oryza sativa.* ; (2) thermodynamic prediction of miRNA–mRNA interactions using ViennaRNA (MFE ≤ -25 kcal/mol) against ribosomal protein and germination-related transcript datasets, followed by GO enrichment analysis; (3) experimental validation by Northern blot across imbibition time points (0, 6, 12, and 24 h) and vegetative tissues; and (4) densitometric quantification with ImageJ and data visualization using Python-based heatmaps.

miRNA sequence retrieval, homology filtering, and target prediction

To identify conserved microRNAs (miRNAs) potentially involved in seed germination in *Zea mays* (maize), we implemented a multi-step bioinformatic pipeline integrating curated databases, cross-species sequence conservation analysis, and functional target prioritization. Mature miRNA sequences from both *Zea mays* and *Oryza sativa* (rice) were retrieved from two major public repositories: the Plant miRNA Database (PMRD; Zhang et al., 2010) and miRBase v22.1 (Kozomara et al., 2019). PMRD provided experimentally supported mature miRNA sequences along with computationally predicted mRNA targets across multiple plant species, while miRBase, which includes both mature and precursor miRNA (pre-

miRNA) sequences, served as a complementary resource for homolog detection and cross-validation.

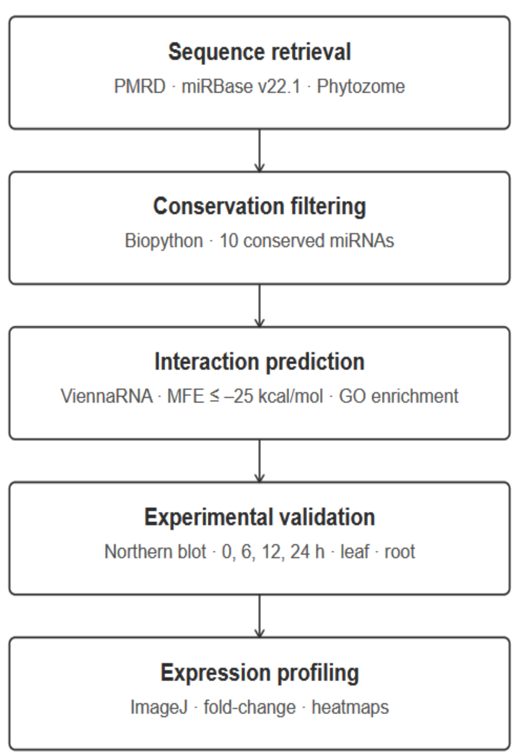

Figure 1. Overview of the methodological pipeline.

For each species, miRNA identifiers and sequences were extracted in text format and used as input for comparative analysis. Pairwise sequence alignments between maize and rice miRNAs were automated using the Biopython library (Cock et al., 2009) within a Google Collaboratory environment. For the PMRD dataset, which contains mature miRNA sequences, we applied a strict conservation filter, retaining only those miRNAs exhibiting 100% sequence identity between *O. sativa* and *Z. mays*. This ensured high-confidence evolutionary conservation at the functional (mature) level. For miRBase, which includes pre-miRNA sequences, we applied a slightly relaxed criterion, retaining pre-miRNAs with ≥85% sequence similarity, a threshold reflecting potential functional conservation despite structural divergence in precursor hairpins. From this filtered set, we curated a final panel of 10 highly conserved miRNAs based on two criteria: 1) Presence in both PMRD and miRBase datasets, satisfying the respective conservation thresholds. 2) Literature-supported roles in seed germination, embryogenesis, or early developmental transitions in plants.

To prioritize biologically relevant targets for downstream analysis in maize, we first mined all experimentally predicted rice mRNA targets associated with these 10 conserved miRNAs from PMRD. Rice gene identifiers (e.g., LOC_Os02g41800.1) were mapped and annotated using the Phytozome platform (Oryza sativa v7.0, Phytozome genome ID: 323, NCBI taxonomy ID: 39947). Given the high sequence conservation of both miRNAs and their targets between rice and maize, particularly for core developmental and ribosomal genes, the rice target list was used to inform and guide our subsequent in silico target prediction in maize. This cross-species approach leverages the depth of functional annotation in rice to overcome limitations in maize target databases. To further refine the candidate list, we performed keyword-based filtering of target gene annotations using terms such as "germination," "seed," "embryo," "meristem," and "embryogenesis." This step prioritized miRNA-target interactions most likely to be functionally relevant during early developmental transitions. The resulting high-confidence miRNA set, enriched for germination-associated targets, was then used for expression profiling in maize via Northern blot and for computational prediction of interactions with maize ribosomal protein (RP) mRNAs, as described later.

Gene Ontology (GO) enrichment analysis

For functional annotation of predicted mRNA targets, we first mapped rice gene identifiers to known functional domains using the Phytozome platform (https://phytozome-next.jgi.doe.gov). To gain insight into biological processes associated with germination, we conducted Gene Ontology (GO) enrichment analysis using the agriGO v2.0 toolkit (Tian et al., 2017). The singular enrichment analysis (SEA) module was employed with default settings, applying Fisher's exact test and a false discovery rate (FDR) correction to control for multiple testing. GO terms with adjusted p-values ≤ 0.05 were considered significantly enriched. The results were visualized through bar charts and directed acyclic graphs (DAGs), which highlighted overrepresented categories in biological processes, molecular functions, and cellular components. This approach provided a functional overview of conserved miRNA targets and enabled the identification of germination-relevant pathways. This enabled further filtering of miRNA-target interactions likely to participate in germination control. The final panel of conserved miRNAs with validated germination-relevant targets was selected for downstream expression profiling in *Z. mays* using northern blot analysis, including comparisons between imbibed seeds and vegetative tissues.

Prediction of germination-related miRNA–mRNA interactions

To evaluate the regulatory role of germination-related microRNAs (miRNAs) in *Zea mays*, we analyzed their interactions with two mRNA groups: ribosomal protein (RP) mRNAs and germination-associated transcripts. RP mRNAs were retrieved from GenBank using the query: ribosomal protein[All Fields] AND "Zea mays"[porgn] AND (plants[filter] AND biomol_mrna[PROP]), yielding 2,084 sequences. A second dataset comprising 33,332 germination-related mRNAs was obtained using a similar query with the keyword germination. Validated Zea mays miRNAs, previously selected through conserved sequence alignment with Oryza sativa using PMRD and miRBase databases (Zhang et al., 2010; Kozomara et al. 2019), were used for interaction analysis. All miRNA and mRNA sequences were processed in Python using pandas and Biopython (Cock et al., 2009). The thermodynamic interaction stability was estimated using the RNA duplex-folding algorithm from the ViennaRNA package (Lorenz et al., 2011), which computes minimum free energy (MFE) values for miRNA–mRNA pairs. Lower MFE values (-25 to -30 kcal/mol) reflect more stable interactions and suggest stronger binding potential. The resulting interaction data, including miRNA IDs, mRNA IDs, and MFE values, were exported for statistical and visualization analyses.

Plant material preparation

Quiescent seeds from *Zea mays* L. cv "tuxpeño" were imbibed and manually dissected to obtain embryonic axes (Rincon-Guzmán et al., 1998). Seed imbibition was conducted by placing the seeds over moisturized cotton with deionized water in the dark, at 27±1 ˚C. On the other hand, six-week "tuxpeño" maize plants from the greenhouse were used to obtain leaf and root tissue for RNA extraction (Bai et al., 2020).

RNA isolation and membrane transfer

Total RNA was extracted from maize embryonic axes at 0 h (quiescent seed), 6 h, 12 h, and 24 h of imbibition, as well as from leaf and root tissues, using TRIzol® reagent (Invitrogen) following the protocol described by Reyes et al. (2007). For miRNA detection, 15 µg of total RNA was resolved on a 15% polyacrylamide gel containing 8 M urea. Electrophoresis was performed in 0.5× TBE buffer using a mini-gel system (1.5 mm thickness), with a 30-minute pre-run at 40 mA followed by sample separation at 20 mA for 1 hour. RNA was transferred onto Hybond-N+ nylon membranes (GE Healthcare) using a semi-dry transfer system (Bio-Rad) at 40 mA for 40 minutes. Membranes were UV crosslinked using a calibrated UV crosslinker at 120 mJ/cm² for two consecutive pulses to ensure efficient nucleic acid immobilization. Transfer efficiency and loading uniformity were verified by post-transfer staining of membranes with 0.2% (w/v) methylene blue (Sigma-Aldrich, Cat. No. M9140) for 15 minutes, followed by gentle destaining with sterile water until ribosomal RNA bands were clearly visible (Reyes & Chua, 2007).

Northern blot

We determined expression levels from miRNA using the transferred membranes blotted with probes made from oligos with the exact complementary sequence for the miRNA candidates. Therefore, the miRNA probes were the anti-miRNA of the original mature miRNA. In this case, oligos of 20 nt were designed to serve as the detection probe. These oligos were labeled using a phosphorylation reaction. T4 Kinase (New England Biolabs, Inc., Ipswich, MA, EUA) was used to incorporate γ-ATP (P32) into the primer sequence. The reaction was incubated at 37 °C for 1 h. The nylon membrane was pre-warmed at 42 °C using ULTRAhyb©-Oligo hybridization solution (Ambion). Following the addition of the radiolabeled oligo-P32 probe to the nylon membrane, hybridization was conducted overnight (Donayre-Torres et al., 2009). Membrane washes were applied to the membrane with the washing solution (2X SSC / 0.5% SDS). Finally, the membrane was exposed using an autoradiography Phospho-imager cassette (Wang et al., 2023).

Visualization of computational predictions and expression profiling of miRNA–mRNA interactions

As mentioned, predicted miRNA–mRNA minimum free energy (MFE) thresholds set at ≤ –25 kcal/mol define stable interactions. MFE values were compiled into pivoted matrices with miRNAs represented along rows and RP transcripts along columns. Heatmaps were generated in Python using the seaborn and matplotlib libraries (Hunter, 2007; Waskom, 2021), applying a viridis colormap to encode binding strength, where darker tones indicated more stable interactions. This enabled visualization of preferential miRNA affinities toward RP transcripts and identification of potential regulatory hubs. For expression profiling, we used ImageJ software v1.53 (Schneider et al., 2012), each miRNA band intensities were measured by selecting regions of interest (ROIs) calculating integrated pixel density values. To correct for loading variation and transfer efficiency, all miRNA signal intensities were normalized to the corresponding methylene blue–stained rRNA band intensity from the same

lane, serving as an internal loading control. Normalized intensities were then expressed in arbitrary units (A.U.) relative to the dry seed (0 h) sample, which was set as the baseline (1.0-fold) for fold-change calculations. Expression levels of conserved miRNAs were evaluated at 6 h, 12 h, and 24 h post-imbibition, as well as in maize root (MR) and maize leaf (ML), and normalized to dry seed (0 h) as the control. Fold-change values relative to 0 h were calculated, averaged across replicates, and corrected for background noise. Heatmaps were generated with seaborn, using a red–blue gradient where red indicated upregulation, blue indicated downregulation, and white represented no change. This visualization revealed distinct dynamic expression trajectories of conserved miRNAs across germination stages and vegetative tissues.

3. Results and discussion

Identification of conserved miRNAs associated with germination

Through homology-based filtering of curated plant microRNA databases, we identified 10 conserved miRNAs shared between *Zea mays* and *Oryza sativa* (Table 1). These miRNAs were retrieved from both PMRD and miRBase v22.1, with stringent criteria applied to select only those mature miRNAs displaying 100% sequence identity (PMRD) or pre-miRNAs with ≥85% similarity (miRBase). The intersection of both datasets, followed by manual curation supported by literature on germination-associated functions, resulted in a robust set of candidate regulatory miRNAs. This cross-species conservation supports their potential involvement in core developmental processes, such as seed germination, which are known to be tightly regulated at the post-transcriptional level by miRNAs (Nonogaki, 2010; Tang et al., 2012).

GO enrichment confirms functional themes relevant to germination

The complete collection of rice mRNA target annotations from the PMRD database was used to retrieve the predicted targets for the 10 conserved miRNAs identified in this study (Supplementary Material 1). Later, we retrieved mRNA targets enriched in biological processes such as embryogenesis, meristem activation, and seed metabolism. These processes are essential during the transition from dormancy to active seedling growth, highlighting the relevance of the selected miRNAs. Gene identifiers (e.g., LOC_Os02g41800.1) were assigned according to Phytozome annotations, as described in the Methods section. These targets were filtered using germination-related keywords to narrow the gene list. Among the identified targets, only miR169a, miR414, and miR415 were found to regulate mRNAs encoding ribosomal proteins. Specifically, miR169a targets ribosomal protein S12 mRNA; miR414 targets ribosomal protein L18p/L5e, L4, and S9-2; and miR415 targets ribosomal protein S18. Additionally, miR160a and miR408 target auxin response factors (ARFs), while miR164 is predicted to regulate genes involved in meristem maintenance and seed maturation. These observations align with previous findings demonstrating the role of miRNAs in controlling developmental checkpoints during germination, particularly via hormone signaling and cell cycle regulation (Nonogaki, 2010; Pan et al., 2018; Reyes & Chua, 2007; Curaba et al., 2014).

To elucidate the biological roles of miRNA targets during rice seed germination, we performed Gene Ontology (GO) enrichment analysis with AgriGO platform, which revealed significant overrepresentation of functions essential for metabolic activation and developmental reprogramming. Highly enriched terms included proteolysis (GO:0006508, $p = 5.5 \times 10^{-13}$), aspartic-type peptidase activity (GO:0070001, $p = 5.0 \times 10^{-12}$), and endopeptidase activity (GO:0004175, $p = 4.4 \times 10^{-10}$), highlighting the importance of controlled protein degradation during the transition from dormancy to active growth. In addition, enrichment of copper ion binding (GO:0005507, $p = 2.0 \times 10^{-15}$) and gene expression (GO:0010467, $p = 9.3 \times 10^{-11}$) underscores the involvement of redox regulation and transcriptional control in early germination (Supplementary Material 1).

Table 1
Conserved miRNAs with seed-specific expression were analyzed during maize germination

miRNA	Sequence	Seed-specific expression
zma-miR160a / osa-miR160a	UGCCUGGCUCCCUGUAUGCCA	N
zma-miR164a / osa-miR164a*	UGGAGAAGCAGGGCACGUGCA	N
zma-miR168a / osa-miR168a	UCGCUUGGUGCAGAUCGGGAC	N
zma-miR169a / osa-miR169a*	CAGCCAAGGAUGACUUGCCGA	N
zma-miR396a / osa-miR396a*	UUCCACAGCUUUCUUGAACUG	Y
zma-miR397a / osa-miR397a	UCAUUGAGCGCAGCGUUGAUG	Y
zma-miR408 / osa-miR408	CUGCACUGCCUCUUCCCUGGC	Y
osa-miR414	UCAUCCUCAUCAUCAUCGUCC	Y
osa-miR415	AACAGAACAGAAGCAGAGCAG	Y
zma-miR528 / osa-miR528	UGGAAGGGGCAUGCAGAGGAG	Y

miRNAs were identified through bioinformatics analysis and experimentally validated using Northern blot. Abbreviations: N = Not expressed in seed; Y = Expressed in seed. (*) highly conserved miRNAs.

These results indicate that conserved miRNAs in rice are likely key regulators of proteolytic activity, oxidative signaling, and transcriptional modulation, supporting their central role in reprogramming the seed transcriptome for successful germination.

Interaction of conserved miRNAs with ribosomal protein-encoding mRNAs

To assess whether the 10 conserved miRNAs preferentially target ribosomal protein (RP) mRNAs, components increasingly recognized for regulatory roles beyond translation, we conducted thermodynamic interaction analysis using the ViennaRNA package. A total of 2,084 RP mRNAs and 33,332 germination-related mRNAs (Figure S1, Supplementary Material 2) were retrieved from GenBank and screened for stable miRNA–mRNA duplex formation (MFE ≤ –25 kcal/mol) (Figure 2 and Figure S2 of Supplementary Material 2). Among all tested miRNAs, miR408 and miR160a exhibited the strongest overall targeting potential, interacting with 42.85% of ribosomal mRNAs and 27.84% of germination-related mRNAs, respectively (Figure S1, Supplementary Material 2). These results underscore the prominent regulatory scope of these two highly conserved miRNAs during early seed development. Functionally, miR408 is known to regulate copper homeostasis, oxidative stress responses, and cell wall remodeling, and is involved in modulating gibberellin and abscisic acid signaling, which are critical hormonal pathways during germination (Gao et al., 2022; Balyan et al., 2023; Huang et al., 2024). Similarly, miR160a plays essential roles in embryogenesis, root development, and auxin-responsive gene expression by repressing auxin response factors, and has been shown to modulate germination and stress resilience (Lin et al., 2018; Guo et al., 2022; Zhou et al., 2021). Together, these findings suggest that ribosomal mRNAs represent not only structural components of translational machinery but also post-transcriptional regulatory hubs during seed germination, mediated in part by conserved miRNAs with dual functions in metabolic and developmental regulation (Martinez-Seidel et al., 2020; Xiong et al., 2021). Thermodynamic interaction analysis revealed that conserved miRNAs preferentially targeted ribosomal protein (RP) mRNAs (Figure 2 and Figures S1 and S2 of Supplementary Material 2). miR408 and miR160a showed the highest targeting frequency and most stable predicted duplexes, highlighting ribosomal transcripts as central post-transcriptional hubs during germination. Several RP genes (e.g., RPL18, RPS7) were recurrently targeted, suggesting selective regulation of both large and small ribosomal subunits. Beyond their canonical structural roles, ribosomal proteins are increasingly recognized for non-canonical functions in stress responses and developmental reprogramming (Martinez-Seidel et al., 2020; Xiong et al., 2021). These findings suggest that conserved miRNAs not only modulate translation capacity but also coordinate ribosome-linked regulatory networks during germination.

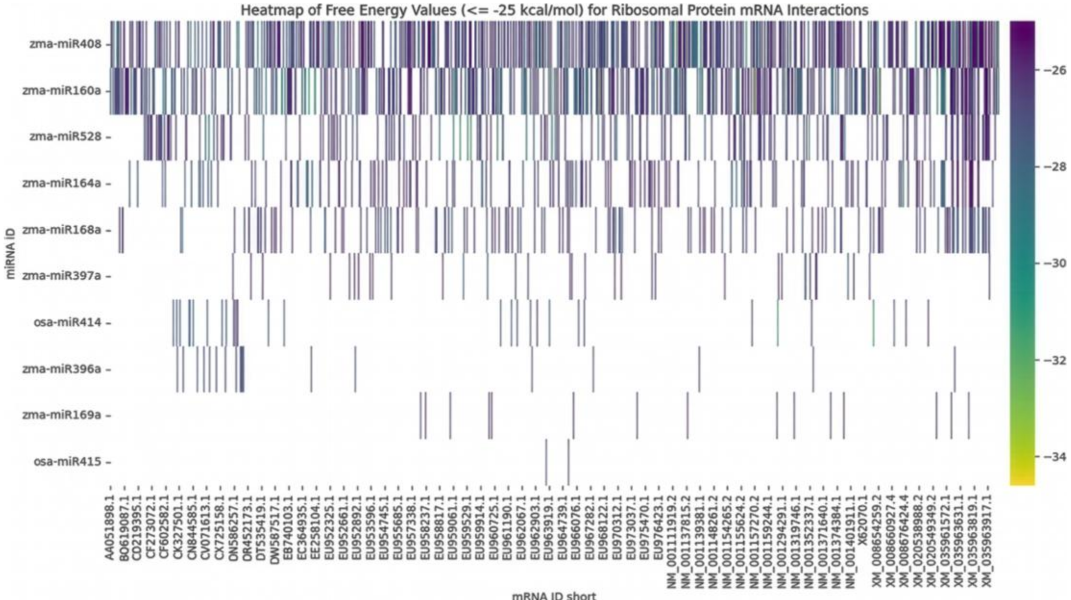

Figure 2. Heatmap of miRNA–ribosomal protein mRNA interactions. Heatmap of free energy values (MFE ≤ –25 kcal/mol) for predicted interactions between conserved maize and rice miRNAs and the dataset of 2,084 ribosomal protein mRNAs retrieved from GenBank. Rows represent individual miRNAs and columns represent ribosomal protein mRNAs, with color intensity indicating interaction strength (more negative values = stronger interactions). Among all tested miRNAs, zma-miR408 and zma-miR160a exhibited the strongest overall binding affinities, with duplexes reaching MFE values as low as –34.6 kcal/mol, highlighting their potential as primary regulators of ribosomal protein transcripts.

Northern blot analysis

To validate the biological relevance of conserved miRNAs, Northern blot hybridization was performed on nine candidates across germination stages (0, 6, 12, 24 h) and vegetative tissues (leaf and root) (Figure 3). Expression profiling revealed distinct temporal and spatial patterns. miR528, miR396a, miR414, miR415, and miR408 showed prominent signals in seed tissues, while miR160a, miR164a, and miR168a exhibited stronger accumulation in vegetative organs. miR169a displayed relatively consistent expression across all samples, suggesting constitutive regulation. Notably, miR528 and miR396a were highly abundant in dry seeds (0 h) and remained elevated throughout early germination, with signals sharply declining or absent in leaf and root tissues indicating seed-enriched, germination-sustained expression. In contrast, miR408 and miR415 were predominantly detected in dry and early-imbibed seeds (0 – 6 h), with expression diminishing by 12–24 h and becoming undetectable in vegetative tissues. This transient, seed-restricted pattern suggests these miRNAs may function in early transcriptional or translational resetting during the transition from quiescence to active growth. These expression patterns align with predicted targeting of ribosomal protein mRNAs and underscore the role of miRNA-mediated regulation in modulating the translational machinery during the seed-to-seedling transition (Sarkar Das et al., 2018).

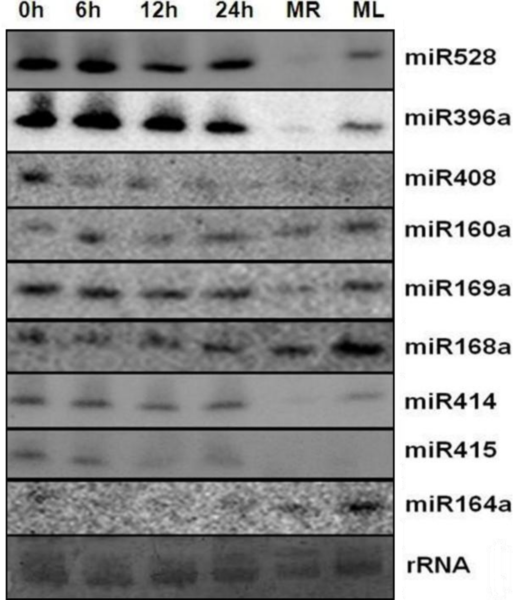

Figure 3. Expression profile of miRNAs in early germination. Northern blot analysis of maize conserved miRNAs from embryonic axes during germination. Germination points of 0h (quiescent seed), 6h, 12h, and 24h were analyzed in comparison to vegetative tissues from maize leaf (ML) and maize roots (MR). As a loading control, methylene blue staining of rRNA is shown.

The quantitative heatmap in Figure 4 further refines these observations by illustrating fold-change dynamics relative to dry seed (0 h). Among all profiled miRNAs, miR160a exhibited the most pronounced and sustained upregulation, increasing steadily from 6 h to 24 h (peaking at 2.59-fold), and remaining elevated in leaf tissue. This progressive induction is consistent with its established role in regulating auxin response factors (ARFs) that drive meristem activation, hypocotyl elongation, and seedling establishment (Nonogaki, 2010; Gutierrez et al., 2012). Far from being "vegetative-specific," miR160a appears to be germination-induced, with its expression ramping up as the seedling emerges, likely preparing the plant for post-germinative growth. Similarly, miR168a showed moderate upregulation during late germination (12 – 24 h) and strong expression in leaf tissue, aligning with its conserved function in modulating AGO1 and maintaining miRNA homeostasis in actively developing tissues (Vaucheret et al., 2006).

In contrast, miR408, while clearly detectable in dry seed, progressively downregulated during imbibition (0.5-fold by 24 h) and nearly absent in vegetative tissues. This pattern of early seed abundance followed by repression is consistent with studies in Arabidopsis showing miR408 downregulation in response to light and oxidative cues during germination (Pan et al., 2018; Ma et al., 2015). Its early peak may reflect a role in managing redox balance or stored mRNA translation during the initial hydration phase. miR528, a monocot-specific miRNA, maintained relatively stable levels during germination but was significantly downregulated in root and leaf tissues, reinforcing its seed-enriched character and potential role in embryonic metabolism or stress buffering, as previously observed in maize and rice (Ding et al., 2023; Wang et al., 2011). The heatmap also reveals that miR415, miR414, and miR164a are consistently downregulated after the dry seed stage, suggesting these miRNAs may act as "brakes" released upon imbibition to permit the derepression of growth-promoting targets.

Together, these data highlight the fine-tuned, stage-specific control of miRNAs during maize development. They provide experimental validation of bioinformatic predictions and significantly enhance our understanding of how post-transcriptional regulators orchestrate one of the plant's most critical developmental transitions. These results not only support prior transcriptomic analyses (Curaba et al., 2014; Zhang et al., 2009) but also offer a prioritized set of miRNAs, particularly the dynamically regulated miR408, miR414, miR415, and the strongly induced miR160a, for future functional studies aimed

at improving seed vigor and stress resilience. Two major expression trajectories emerged: i) Early-peaking, seed-restricted miRNAs: miR408 and miR415 are highly abundant in dry seed but rapidly decline during germination, suggesting roles in the earliest molecular events of imbibition. ii) Germination-induced miRNAs: miR160a and miR168a increase progressively during germination, likely supporting seedling morphogenesis and regulatory network maturation.

Interestingly, miR414 and miR415, while present in rice databases, had not been previously experimentally detected in maize, highlighting them as novel candidates for functional characterization in this species. While miR160a, miR164a, and miR168a are classically associated with vegetative development and hormone signaling (Dong et al., 2022; Tang et al., 2012), our data show that miR160a, in particular, is strongly induced during germination, suggesting its regulatory role begins earlier than previously assumed, likely bridging seed activation and seedling growth. miR164a and miR168a, though detectable in seeds, show peak accumulation in leaves, indicating their primary functions manifest post-germination. The functional significance of these dynamics is underscored by known roles: miR408 regulates ROS and promotes germination via PLANTACYANIN repression (Jiang et al., 2021); miR528 responds to imbibition and drought (Li et al., 2013); miR396 promotes cell division via GRFs (Ding et al., 2012); and miR160 fine-tunes ABA sensitivity via ARF10 (Dong et al., 2022). Even miR156 and miR159, though not profiled here, are known to influence seed size and grain weight via SPL and MYB factors (Zhang et al., 2013; Wang et al., 2023), linking early miRNA activity to agronomic outcomes. Notably, miR414 and miR415 exhibit tightly regulated, transient expression, detectable only in early germination stages. Though their targets remain uncharacterized, their dynamics suggest involvement in hormone crosstalk or the clearance of maternal transcripts. These findings collectively reveal a temporal choreography of miRNA activity, with distinct modules operating in dry seed, during imbibition, and in emerging seedlings, underscoring the precision of post-transcriptional control in the dormancy-to-growth transition. miRNAs such as miR160a, miR164a, and miR169a, which are classically linked to auxin signaling and stress adaptation in vegetative tissues (Dong et al., 2022; Tang et al. 2012), showed minimal or no expression during germination, indicating their post-embryonic specialization. Notably, miR414 and miR415 exhibited transient and germination-enriched expression patterns. Although their specific targets remain uncharacterized, their dynamic regulation suggests a potential role in early transcriptional resetting or hormone crosstalk. These findings collectively point to a temporal partitioning of miRNA activity between seed and vegetative phases, underscoring the developmental precision of post-transcriptional regulation during the transition from dormancy to active growth. Conserved microRNAs profiled during maize seed germination are summarized in Table 2.

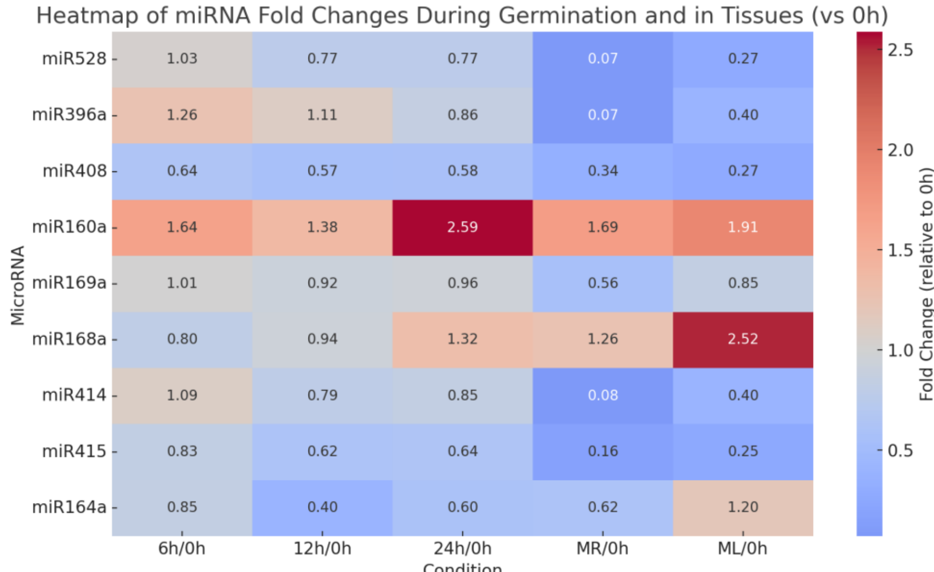

Figure 4. Dynamic Expression Profiles of Conserved miRNAs During Maize Seed Germination and in Vegetative Tissues. Heatmap depicting fold-change values for microRNA expression at 6 h, 12 h, and 24 h post-imbibition, as well as in maize root (MR) and maize leaf (ML), all normalized to the dry seed control (0 h). Expression values were derived from densitometric quantification of Northern blot signal intensities. The color scale ranges from blue (downregulation) to red (upregulation), centered at 1.0-fold (no change).

Table 2
Comparative analysis of conserved MicroRNAs in maize germination and vegetative development

miRNA	Reported Role	This Study	Previously Reported Function	Reference
miR160a	ARF10 targeting, ABA signaling	Strong in vegetative tissue	Embryonic axis elongation, auxin regulation	Dong et al., 2022; Sarkar Das, 2018
miR164a	NAC TFs regulation	Detected in leaf/root only	Apical meristem control	Ding et al., 2012
miR168a	AGO1 targeting, RISC feedback	Weak in seed, moderate in vegetative	miRNA biogenesis loop	Guo et al., 2022
miR169a	NFYA targeting, ABA response	Low in germination tissues	Stress regulation	Ding et al., 2012
miR396a	GRFs targeting, cell division	Strong in early seed stages	Seedling meristem initiation	Ding et al., 2012
miR397a	Laccases, brassinosteroid signaling	Not detected	Grain size & wall biosynthesis	Zhang et al., 2013
miR408	ROS signaling, stress adaptation	High at 0 h, declines over time	Redox balance & light response	Song et al., 2018; Jiang et al., 2021
miR414	Unknown	Expressed at 6–12 h only	Novel expression profile	This study
miR415	Unknown	Transient germination signal	Newly observed in maize	This study
miR528	Monocot-specific, developmental repressor	Strong signal in dry/germinating seed	Somatic embryogenesis & stress tolerance	Luján-Soto et al., 2021

4. Conclusions

This study provides a systematic comparative analysis of conserved microRNAs during maize seed germination, integrating cross-species bioinformatics with experimental validation to address the regulatory roles of miRNAs in the seed-to-seedling transition. Through homology-based filtering of PMRD and miRBase v22.1, ten conserved miRNAs shared between *Zea mays* and *Oryza sativa* were identified, confirming that evolutionary conservation at the mature sequence level is a reliable criterion for selecting functionally relevant candidates across monocot species. Thermodynamic interaction analysis revealed that conserved miRNAs preferentially target ribosomal protein (RP) mRNAs over germination-related transcripts in general, with miR408 and miR160a exhibiting the broadest and most stable predicted interactions. This finding supports the central hypothesis of the study: that conserved miRNAs regulate the dormancy-to-growth transition not only through canonical transcription factor targets but also by directly modulating the translational machinery, positioning ribosomal mRNAs as post-transcriptional regulatory hubs during germination. Northern blot profiling across imbibition time points (0, 6, 12, and 24 h) and vegetative tissues revealed distinct temporal expression modules. miR528 and miR396a were sustained throughout seed stages and absent in vegetative tissues, indicating seed-enriched, germination-sustained functions. miR408 was highly abundant in dry seed and declined progressively during imbibition, consistent with a role in early redox balance and stored mRNA management. In contrast, miR160a was progressively induced during germination, reaching peak accumulation at 24 h, which challenges its classification as a strictly vegetative miRNA and implicates it in bridging seed activation and seedling establishment through auxin response factor regulation. Notably, miR414 and miR415, previously undetected experimentally in maize, displayed transient, germination-restricted expression patterns, establishing them as novel candidates for functional characterization in this species. Gene Ontology enrichment analysis of predicted targets confirmed overrepresentation of proteolysis, oxidative signaling, and transcriptional regulation, reinforcing the biological coherence of the identified miRNA–target interactions and their relevance to the metabolic reactivation that defines early germination. Taken together, these results reveal a temporal choreography of miRNA activity in maize germination, with discrete regulatory modules operating in dry seed, during imbibition, and in emerging seedlings.

The data provides a prioritized set of experimentally validated miRNAs, particularly miR160a, miR408, miR414, and miR415, as entry points for functional studies aimed at dissecting post-transcriptional control of the seed-to-seedling transition. Future research should prioritize the functional validation of miR414 and miR415 through loss- and gain-of-function approaches, alongside ribosome profiling experiments to directly test whether miR408 and miR160a modulate translational efficiency of specific RP mRNAs during imbibition. Collectively, the miRNA–ribosome regulatory axis described here opens promising avenues for precision breeding strategies aimed at engineering seed vigor, germination uniformity, and stress resilience in maize and related cereal crops.

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

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