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# Identification of zinc responsive miRNAs using high throughput small RNA sequencing in maize kernel

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**Abstract**

**Zinc (Zn) is an important micronutrient for plants growth and its deficiency affects the normal function of physiological pathways. Development of maize (*Zea mays*) kernel is a complex process that is associated with the expression of many genes, regulated at transcriptional and post transcriptional level. Micro RNAs (miRNAs) are regulatory molecules that play vital roles in almost all aspects of biological processes. Although several miRNAs have been identified in maize, yet their role in maize kernel development not been studied. Here, we have identified 69 known and 44 novel miRNAs using high-throughput small RNA sequencing of 10 days old baby kernel of high zinc containing maize inbred VQL-2, grown under Zn deficient soil. Among these, 33 known and 35 novel miRNAs were found to be differentially regulated. Gene ontology and KEGG pathway analysis of their targets revealed involvement of several essential pathways such as starch and sucrose metabolism, arginine and proline metabolism. This study provides the valuable information about the role of miRNAs and target networks in response to Zn deficiency in maize kernel.**

**Key words:** Kernel development, *Zea mays*, miRNA, qRT PCR, Zn deficiency

## Introduction

Zinc (Zn) is one of a key micronutrient for proper growth and development of plant (Tariq et al. 2015). It is involved in DNA and RNA metabolism, protein synthesis, carbohydrate metabolism, activation of enzymes, formation of pollen, resistance to pathogens, maintenance of membrane integrity and auxin metabolism in cells (Alloway 2008). In plant, Zn deficiency causes white to yellowish leaf, retarded growth, and yield loss (Dobermann 2000) as a result of elevated soil pH, absorption of Zn with other soil particles or precipitation and redox potential condition in soil. Maize (*Zea mays* L.) is a major cereal crop around the world and ranks third highest produced among the all cereals (Singh et al. 2021). As Zn is important micronutrient for human as well as animals, an deficiency of Zn causes human malnutrition and infectious diseases in developing countries (Mukhopadhayay et al. 2013). So, there is need to identify those genes responsible for Zn content in maize kernel.

MicroRNAs (miRNAs) are small, non-coding RNA of length 20-24 nucleotides that function as to cleave the target mRNAs or translational inhibition at post-transcriptional level (Singh et al. 2021). It has been reported to play major role in nutrient deficiency (Paul et al. 2015). For example, miR395 regulates sulphur assimilation and translocation in *Arabidopsis* under low sulphur condition (Kawashima et al. 2009). Shi et al. (2012) reported that, 13 miRNAs were upregulated and only two miRNAs such as miR399b and miR845a were downregulated in Zn deficient *Brassica juncea* roots. Zeng et al. (2019) identified 10 Zn deficiency responsive miRNAs in rice seedlings. In recent years, high throughput sequencing is a widely adopted technique to discover the miRNAs. Using this technique, numerous miRNAs has been identified in maize crop (Gupta et al. 2017). However, there is lack of systematic study on the discovery of Zn responsive miRNA in maize.

The aim of present study was to identify Zn deficient responsive miRNAs, their potential targets

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and functional analysis in developing kernel of maize through high throughput small RNA sequencing techniques. In this study, we discovered known and novel miRNAs analysed their expression levels during developing maize kernel grown under Zn deficient soil. Our result also suggested the networking between miRNAs and their corresponding target genes in Zn deficient condition. Together these findings contribute to the understanding of the regulatory roles of miRNAs to Zn limiting conditions in maize.

## Materials and methods

### *Plant material and treatment*

Seeds of a high Zn content maize cultivar, VQL-2 were collected from ICAR-Vivekananda Parvatiya Krishi Anusandhan Sansthan, Almora, India. Plants which were grown in soil with optimum Zn (7 mg DTPA-Zn/kg) described as control and in Zn deficient soil (0.5 mg DTPA-Zn/kg) described as treated. Young kernels on $10^{th}$ days after pollination were collected from both control as well as treated soil. Samples were frozen immediately in liquid nitrogen and stored at -$80^{o}$ C until RNA extractions.

### *RNA extraction, library preparation and sequencing*

Total RNA was extracted from 100 mg tissue of 10-days-old maize kernels using RNAeasy Plant Mini Kit (QIAGEN, MD). Equal quantities (1 μg) of RNA from three biological replicate samples were pooled to prepare two individual libraries i.e. one control and one treated. The small RNA libraries were prepared using Illumina Trueseq Small RNA kit. After libraries preparation, small RNA sequencing was performed on Illumina HiSeq 2000 platform. All sequencing data had been submitted to NCBI (http://www.ncbi.nlm.nih.gov/) as Bioproject (PRJNA271417), BiosampleSAMN 03274331), and Sequence Read Archive (SRA) database with submission accession number SRX826359.

### *In silico analysis of Small RNAs*

After sequencing, the raw reads were checked for the quality, adapter trimming and primer contamination using Cutadapt 1.14 (Martin 2011). Only high quality reads ranged from 13-28 nt were extracted from adaptor trimming, which was clustered into unique reads in each library and used for further downstream analysis. High quality reads were mapped to the maize genome (Maize B73 RefGen_v3) downloaded from Ensembl Plant (Howe et al. 2020). Subsequently, sRNA mapped clean reads were aligned and BLAST search against Rfam11.0 (Burge et al. 2012) to remove the other non coding RNA sequences like rRNA, tRNA, scRNA, snRNA and snoRNA. Finally, the remaining unannotated reads were kept for identification of miRNAs.

### *Identification of known and novel miRNA*

The unique sRNA reads were aligned to the maize miRNA precursors and mature miRNAs in the miRBase 21.0 database (Kozomara et al. 2019) to identify known miRNAs. Sequences with $\leq$2 mismatch bases were allowed for known miRNAs. After the removal of known sequences from data, the left behind un-annotated sRNAs sequences were used to identify novel miRNAs using miRDeep2 with default parameter (Friedländer et al. 2011).

### *Differential expression of miRNA, its target prediction and functional analysis*

To determine the expression of identified known and novel miRNAs, DEseq2 package was used (Love et al. 2014) with default parameter. The $\log_2$ fold-Change $\geq$ 1 was set for upregulated miRNAs and $\log_2$ fold-Change $\leq$ –1 was for downregulated miRNAs. Targets were predicted for differentially expressed known and novel miRNAs using psRNA Target program with default parameters (Dai et al. 2011). Maize transcripts were used as reference for target search. Further Gene ontology (GO) as well Kyoto encyclopedia of genes and genomes (KEGG) pathways were generated by using BLAST2gO software (Conesa and Götz 2008). The interaction network of miRNAs and their target genes was drawn using Cytoscape software (Agilent Technologies Co., USA).

### *Validation of miRNA through quantitative real time-PCR*

qRT-PCR was performed to validate the miRNA deep sequencing results. Sixteen highly upregulated and downregulated miRNAs were selected for expression study. Approximately 1 μg Small RNAs were extracted from control and treated (Zn deficient soil) maize kernel. Small RNAs were reverse transcribed with Mir-x miRNA First-Strand Synthesis kit (Clontech) following manufacturer's instructions. All the primers for qPCR experiment are listed in Supplimentary Table S1 (for all supplimentary tables go to - Supplimentary data link: https://docs.google.com/spreadsheets/d/e/2PACX-1vTYZ1bqcPj08U3MZhcG1hMlgLhIkW3CDJ7A_JmIImNGGR0ysb_xwth8rUUaapnRxkql6b2wY1yM2gS_/pubhtm). After confirming the

sequence, SYBR Advantage qPCR Premix (Clontech) was used for qRT-PCR analysis using Roche 454 qPCR system (Roche, USA) as described previously (Mondal et al. 2015). The relative expression of miRNA was calculated using $2^{-\Delta\Delta ct}$ method (Mondal et al. 2018). U6 snRNA was used as internal control primer for miRNA. All reactions were conducted with 3 biological replications each with 2 technical replications.

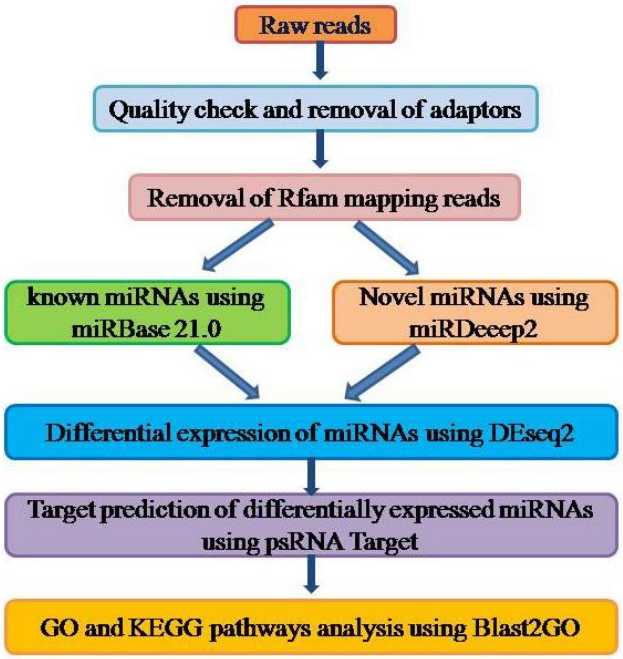


**Fig. 1. Workflow diagram of miRNA discovery and analysis in maize**

**Table 1.** Sequencing reads of control and treated sRNAs libraries

| Name of the reads | Control | Treated |
|---|---|---|
| Raw reads | 21,676,267 | 13,182,678 |
| High quality | 19,317,457 | 11,308,077 |
| Total clean reads | 18,694,443 | 8,291,289 |
| Clean reads (Unique) | 2,830,353 | 3,287,979 |
| Map to miRbase | 2,17,089(7.67%) | 72,524(2.20%) |
| Known miRNAs | 62 | 56 |
| Novel miRNAs | 33 | 19 |

## Results

### *miRNAs analysis during maize kernel development*

A total of 21,676,267 and 13,182,678 raw reads were generated from control and treated libraries respectively.. The workflow diagram of miRNA analysis is shown in Fig. 1. After adaptor removal, 19,317,457 and 11,308,077 of high quality reads were generated from control and treated library respectively (Table 1). A total number of 18,694,443 (86.24%) clean reads in control and 8,291,289(62.89%) clean reads treated libraries were obtained from mapping with maize reference genome. This represented 2,830,353 and 3,287,987 unique reads in control and treated libraries respectively (Table 1). Majority of sRNAs in libraries were 20-24 nt in length, along with 21nt sRNA the most abundant (Fig. 2). The other types of sRNAs were discarded from both libraries. Finally, a sum of 2,17,089 (7.67%) unique reads in control and 72,524(2.20%) unique reads in treated were mapped to miRBase for known miRNA identification.

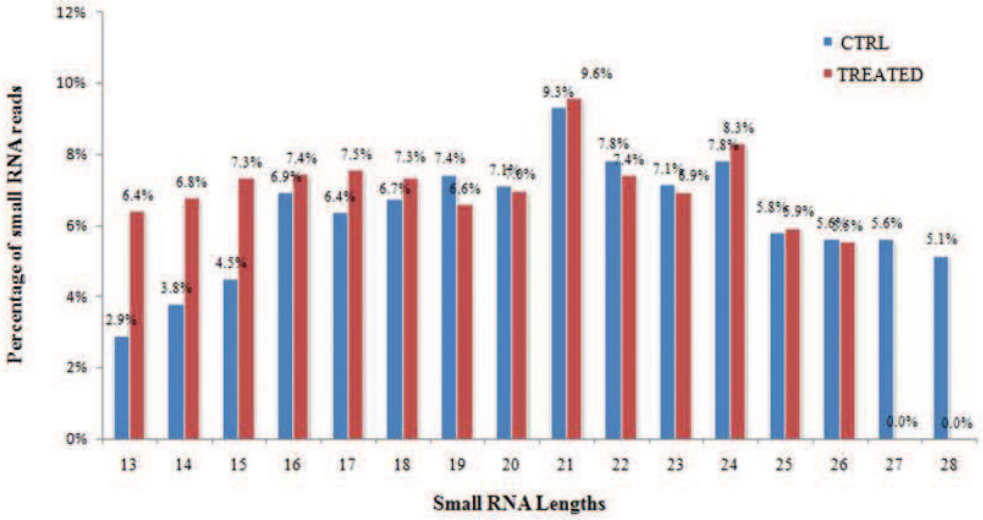


**Fig. 2. Size distribution of sRNA clean reads in maize control and treated libraries**

### *Identification of known and novel miRNAs*

Based on the mapping search, a total of 69 known miRNAs were identified in which, 62 and 56 miRNAs were present in control and treated libraries respectively (Fig. 3a; Supplementary Table S2). These identified miRNAs in control library belong to 23 families, among which, miR168a was highly expressed. Similarly, miRNAs identified from treated library belongs to 26 families along with miR168a family had highest expression (Supplementary Table S2). The remaining unannotated sRNA reads were taken to discover novel miRNAs. A sum total 44 novel miRNAs were discovered including 33 miRNAs in control library and 19 miRNAs were identified in treated library (Fig. 3a; Supplementary Table S3).

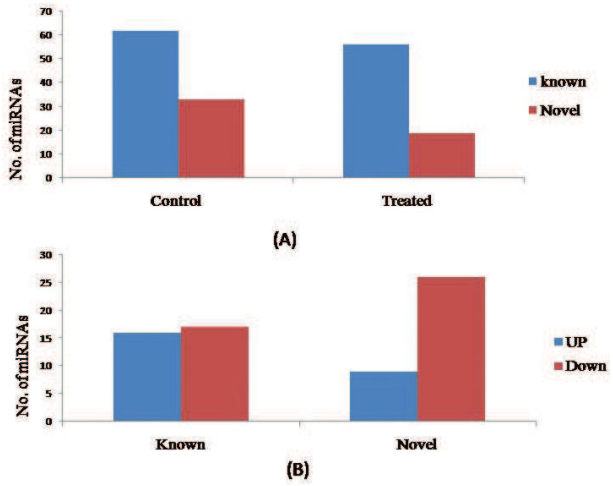


**Fig. 3. Distribution of predicted number and expression of miRNAs in *Zea mays.* A) Number of known and novel miRNAs identified in control and treated library. B) Differentially expressed miRNAs in known and novel sample**

### *Differential expression analysis of known and novel miRNAs*

The analysis showed that, 33 miRNAs from 13 families were differentially expressed in the known Zn deficient maize (Fig 3b; Supplementary Table S4). Out of these, 16 miRNAs were upregulated and 17 miRNAs were found to be downregulated in Zn depletion condition. Among these, miRNAs families i.e. miR156 showed significant result. Similarly we identified 35 novel miRNAs were differentially expressed, including 9 upregulated miRNAs and 26 downregulated miRNAs (Fig. 3b; Table S4). Several novel miRNAs were found to be highly differentially expressed as compared to known miRNAs (Supplementary Table S4).

### *Identification of miRNA target and their function*

From psRNA target, a total 2915 targets were found for 69 known miRNAs and 2128 targets were discovered for 44 novel miRNAs (Supplimentary Table S5). These results suggested that, most of the miRNAs have multiple targets. On the contrary, single gene was also targeted by different miRNAs. These targets include DNA binding domains, hormone signalling, protein kinases, signal pathways, transcription factors and transporters.

GO analysis identified that, known miRNAs targets were involved in 13 biological processes, 46 molecular functions and 3 cellular components (Supplimentary Table S6). Similarly, novel miRNAs were associated with 6 biological processes, 30 molecular functions and 4 cellular components (Supplimentary Table S6). In addition, KEGG pathway discovered 102 and 92 metabolic pathways in which differentially expressed known and novel miRNAs target genes, respectively (Supplimentary Table S7).

### *Validation of miRNAs*

To examine the reliability of the sequencing data, 16 miRNAs including 8 known miRNAs and 8 novel miRNAs were chosen based upon their expression of NGS data for qRT-PCR analysis. Among these, 8 miRNAs (miR159h, miR159k, miR171c, miR171m, novel-miR017, novel-miR022 novel-miR031 and novel-miR037) were upregulated and 4 miRNAs (miR164h, miR162, miR399j and miR026) were downregulated (Fig. 4). We have used same tissue for validation, however, it has been seen that while all the downregulated miRNAs have the same pattern of expression with that of qRT-PCR value yet 1 miRNA i.e miR171m from 4 upregulated, found to have lower expression level with compare to the *in silico* data. Although it is difficult to point out the actual reason, yet it is reported in the literature (Mondal et al. 2015). On the contrary, 3 among the 4 downregulated novel miRNAs did not amplify though all the 4 novel miRNAs were having the same pattern of expression. This could be due to the fact that, novel miRNAs have lower expression level as compared to the conserved miRNAs that is why through qRT-PCR, we failed to detect some of the novel miRNAs.

### *Network analysis*

The network between miRNAs and target genes were elucidated (Fig. 5). As shown in figure, numerous transcription factors were found in target genes including NF-Y (the putative target of miR169c, o,

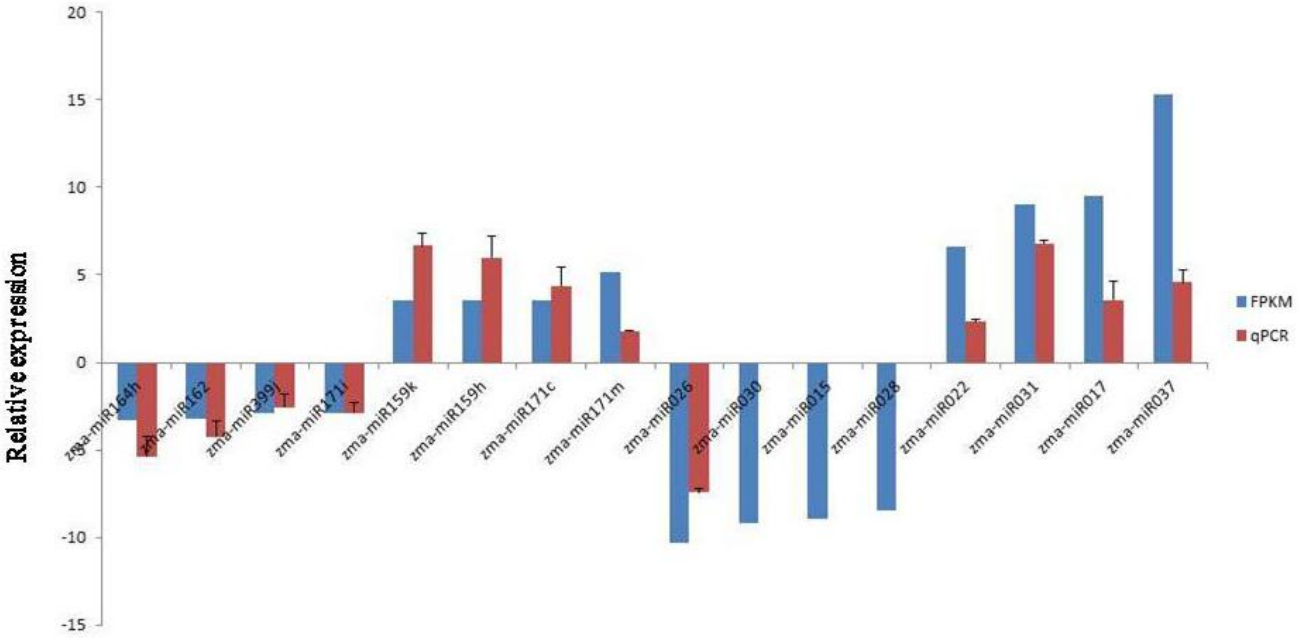


**Fig. 4. Relative expression analysis of 16 miRNAs. Blue colour indicates differential expression in transcriptome data and red colour indicates the relative expression in qRT PCR**

miR171c, f, novel-miR012 and novel-miR030), NAC (miR164e, h, k, miR166k, miR167c, novel-miR013 and novel-miR033), MADS (miR164e,k and miR171f), MYB (miR164h, miR166j, miR171c, novel-miR002, novel-miR025 and novel-miR029) which were involved in development, stress resistant as well as hormonal signal transduction. Apart from that, a membrane protein known as ZINC INDUCED FACILITATOR was targeted by zma-miR166m.

## Discussion

Zn is a vital trace element which affects growth and development of plant (Mukhopadhayay et al. 2013). Now a days, miRNA is known to regulate gene expression in almost every domain of life including nutrient uptake and mobilization under nutrient deficiency. But information of miRNAs in response to Zn deficiency during maize kernel development is still lacking. In present study, we identified and validated Zn deficient responsive miRNAs and their target genes involved in maize kernel development. Here, we discovered 69 known miRNAs and 44 novel miRNAs along with their precursors and target genes in Zn deficient maize young kernel. The control and treated libraries were compared to identify the differential expressed miRNAs in Zn deficient plants. Interestingly, in novel miRNAs, it was found that, highly enriched nov-miR026 and nov-miR037 were also found to be highly differentially expressed. Some of the predicted miRNA families such as miR171, miR398, miR399 and miR408 were studied previously in *Oryza sativa* (Zeng et al. 2019) and *Sorghum bicolor* (Li et al. 2013) which suggest their conserved role in plant response to Zn deficiency.

We also validated the expression of some miRNAs through qRT-PCR and were found to be consistent with the expression of sequencing data. In GO annotation of biological process, highest numbers of miRNAs were found to involve in primary metabolic process, and phosphate metabolic process. In molecular function, it was seen to have maximum catalytic activity, transcription factor activity, kinase activity and transferase activity. Moreover, miRNA target genes were also annotated in multiple KEGG pathways. Among them, starch and sucrose metabolism, arginine and proline metabolism it was seen to have maximum.

Transcription factors are crucial for plant tolerance under nutrient stress. Several differentially expressed miRNAs target like auxin response factor (ARFs), nuclear factor Y subunit (NF-Y) Basic helix loop helix (BHLH) and Teosinte branched1/Cincinnata/ proliferating cell factor (TCP) were involved in nutrient stress (Shi et al. 2013). Auxin is a primary phytohormone which mediates growth and

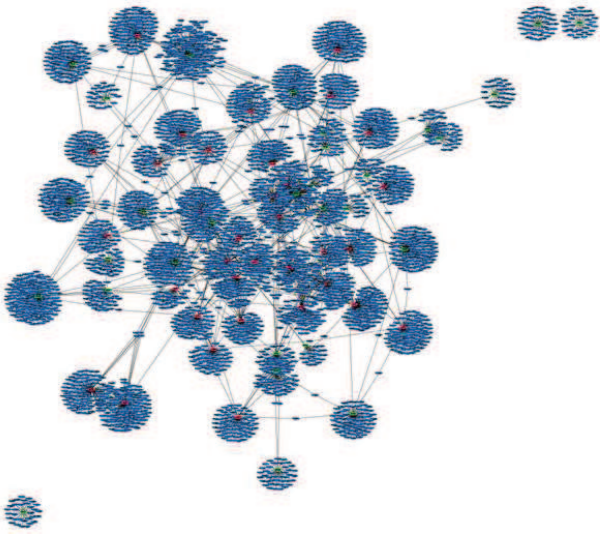

**Fig. 5. The regulatory networking between miRNAs and target genes through Cytoscape. Red colour indicates known miRNAs, green colour indicates novel miRNAs and blue colour indicates target mRNAs**

developmental response in plants. In Zn deficiency, lateral root is formed by inhibiting primary root (Shi et al. 2013). Auxin reception and signal transduction pathways were targeted by transcription factor *ARF*. Here we found miRNA families miR167 and miR399 were upregulated by targeting *ARF*. This result suggests that, the expression of these miRNAs might downregulate the target gene *ARF*. It was also reported that, basic helix loop helix (bHLH) transcription factor family 87 regulates process of grain filling in maize (Zhang et al. 2018). Here, we found that novel-miR87 miR36 were downregulated in Zn deficient plant targeting bHLH87. This suggests it might regulate targets in nutrient deficient state. miR169 was repressed by N, P and S deficiency in *Arabidopsis* (Hsieh et al. 2009; Zhao et al. 2011). So this miRNA may play specific role in nutrient homeostasis. Here, miR169 was also found to be downregulated in Zn deficiency targeting NUCLEAR TRANSCRIPTION FACTOR Y (NF-Y). Also, we got repression of novel miRNA novel-miR30 with target gene NF-Y. This suggests that, miRNA 169 might play some regulatory role in Zn deficiency.

Transporters play major roles in regulation of nutrient homeostasis. It has been reported that, ZINC INDUCED FACILITATOR (*ZIF*) gene and the two other ZINC INDUCED FACILITATOR-LIKE gene (*ZIFL1 and ZFL2*) form a distinct membrane protein family that involve in regulation of Zn homeostasis in *Arabidopsis* (Haydon and Cobbett 2007). The *ZIFL* gene believed to play vital role in Zn translocation to seeds. In present study, we found upregulation of miR395, targeting *ZFL1 gene*. This miRNA may regulate the *ZIFL* gene in Zn deficiency. Zn stress increases superoxide dismutase (SOD) and peroxidase (POD) activity however, decreases catalase (CAT) activity that were observed under conditions of Zn stress (Cui and 2011). In present study, we found that miR408b, novel-miR015 and novel-miR025 were downregulated under Zn stress targeting peroxidase. It suggests that, peroxidase might be upregulated by decreasing the expression of miRNAs. In contrast, miR166n was found to be induced under stress through the target catalase. It indicates that, probably there is downregulation of target gene through induction of miRNA.

The primary response of nutrient stress is to generate ROS enzyme. So, result showed miR408, miR164b and miR162 may play an important role to nutrient stress as their targeted enzymes involved in oxidation-reduction reaction. Nutrient stress also decreases plant immunity to biotic stress. In our study, miR159h and nov-miR014 were differentially expressed under nutrient stress, which targeted leucine-rich repeat (LRR) protein, Jasmonic acid-amidosynthetase (JAR1). This showed that, presence of common regulatory mechanism between biotic and abiotic stress. So, nutrient stress may make plant susceptible to various diseases.

Finally, few uncharacterized protein coding genes with unknown functions were targeted by known and novel miRNAs. These proteins may be specific to maize. Although, these type of targets were well reported in literature before, but it will be fascinating to reveal their actual functions in Zn deficient maize. The identification and functional analysis of known and novel miRNAs would bring opportunity in future to exploring Zn response mechanisms in plants and regulation by miRNAs. Further research could uncover more details about the functions of these miRNAs in Zn deficiency.

**Authors' contribution**

Conceptualization of research (TKM); Designing of the experiments (TKM); Contribution of experimental materials (TKM); Execution of field/lab experiments and data collection (AKP, JN); Analysis of data and interpretation (AKP, AD, TKM); Preparation of

manuscript (TKM, AKP).

**Declaration**

The authors declare no conflict of interest.

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