

Regulation of MAP kinase signaling cascade by microRNAs in *Oryza sativa*

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Mitogen activated protein kinase (MAPK) pathway is one of the most conserved signaling cascade in plants regulating a plethora of cellular processes including normal growth and development, abiotic and biotic stress responses. The perception of external cues triggers the phosphorylation of three tier MAPKKK-MAPKK-MAPK cascade which finally modifies a downstream substrate thereby regulating the cellular processes. Whereas, the transcription regulation by MAPKs, mediated through their substrates is well studied in plants, the transcription and post-transcriptional regulation of the MAPK genes are poorly understood. Previous studies from the animals systems suggested the miRNAs regulate the post-transcriptional regulation of MAPK transcripts. Here we attempt to unravel the post-transcriptional regulation of MAPKs by miRNAs in model crop plant *Oryza sativa*. Using *in silico* tools, we predict the miRNAs for 98 out of 99 MAPK transcripts. The predicted miRNAs were validated for the biological relevance of their function. The inverse correlation between relative transcript levels between the MAPKs and their predicted miRNAs validated the *in silico* prediction. Taken together, this report demonstrates the significance of miRNAs in regulation of the MAPK pathway in plants with a new direction to study the plant signaling molecules.

Introduction

MAP kinases belong to the CMGC (CDK, MAPK, GSK-3, and CKII) group of the protein kinase superfamily and are extensively involved in various stress responses in all the eukaryotes.^{1,2} Since plants are sessile, they possess a large number of MAP kinase family members to efficiently combat stress conditions. A typical MAP kinase cascade (MAPK cascade) consists of a MAPKKK (MKKK/MEKK/MAP3K) which is phosphorylated and activated by its upstream receptor and in-turn phosphorylates and activates its downstream MAPKK (MKK/MEK/MAP2K). The activated MAPKK then phosphorylates and activates a MAPK (MPK), which ultimately transfers the signal to its specific nuclear or cytoplasmic substrates. Many a times the MAPKs may move into the nucleus to transfer the signals in the form of phosphate groups. The model plant *Arabidopsis thaliana* consists of 80 MAPKKKs, 10 MAPKKs and 20 MAPKs.^{3,4} Clearly, in *Arabidopsis* the signals perceived by 80 MAPKKKs must converge into 10 MAPKKs which is possible by the interaction of each MAPKKs with multiple MAPKKKs. It is also obvious that 20 MAPKs being encoded by the *Arabidopsis* genome, more than one MPK interacts with a single MAPKK. The same is the case in the model crop plant rice, where the genome encodes 75 MAPKKKs, 8 MAPKKs and 16 MAPKs.^{4,5}

One of the interesting functions of the MAP kinase cascade is the regulation of microRNA biogenesis in humans. MicroRNAs are 21–24 nucleotide smallRNAs molecules that are

extensively involved in gene regulation. MKK1-Erk2 signaling phosphorylates TRBP (a dsRNA binding protein), which associates with Dicer to form functional miRNA generating complex.⁶ In plants, a dsRNA binding protein HYL1 associates with DCL1 to form a functional miRNA generating complex.⁷ Similar kind of regulation can be anticipated in plants considering the functional conservation of the MAP kinase cascade.

Owing to their large number, size, heterogeneous structure and their limited substrate specificity,⁸ the knowledge about the functions of MAPKKKs is limited. Out of the 80 MAPKKKs encoded by the *Arabidopsis* genome only a few of them are functionally characterized. The vast repertoire of MAPKKKs encoded by the plants suggest their diversity and specificity in recognizing the external signals. Therefore, understanding the roles of MAPKKKs and their regulation will complete the knowledge of the whole MAPK cascade itself. Recent studies report the development of artificial microRNA (amiRNA) that specifically targets a MKKK causing reduced sensitivity to ABA during seed germination as compared with wild type.⁹ Studies from animal systems, especially in *C. elegans* have clear evidences showing that MAPKKK transcripts are targets of miRNA mediated regulation.¹⁰ Studying the similar kind of regulation in plants would reveal exciting details about the conservation of the two major regulatory pathways and the underlying nexus in plant and animal kingdoms. To uncover the functional miRNA mediated-MAP kinase transcript regulation in plants, we chose very important and economic model crop plant rice. In the present study,

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we predicted the microRNAs targeting rice the three different components MAP kinase cascade transcripts. A single microRNA was identified to target eight different MAPKKK transcripts thereby regulating the complete downstream MAP kinase signaling cascade.

Results

Identification of the potential miRNAs targeting transcripts of components of MAP kinase cascade

Among several tools available online for miRNA target predictions, only a few can be used for the prediction of targeting miRNAs from the target transcript sequence. One such tool is Microinspector¹¹ applicable to the plant systems as well. The online tool Microinspector accepts the user specified DNA/RNA sequence to identify the potential miRNA binding sites from the list of the miRNAs provided. The species can be chosen from the list to specify the optimum hybridization temperature and binding energy for the miRNA-target pair. The miRBase release 17 consisted of 452 mature miRNAs from *Oryza sativa*. All the mature miRNAs from rice were downloaded and submitted each time along with a single MAP kinase coding sequence to the Microinspector tool. The results obtained were saved for each of the MAP kinase transcript for further analysis.

A total of 99 sequences corresponding to 16 MAPKs, 8 MAPKKs and 75 MAPKKKs were submitted for the identification of potential miRNA binding sites and corresponding miRNAs. Among several predicted miRNAs for a single transcript, only one miRNA displaying the least free energy was selected for

a particular transcript. For all the members of 99 MAP kinase cascade component sequences analyzed, the miRNAs for 98 members were predicted except for MKK3 (Supplementary Table 2). Interestingly, it was observed that a single miRNA, miR531 is targeting most of the members of MAPK cascade gene family (25 out of 98). Additionally, by using the target search function of PMRD, miR1429_5p was found to potentially target MPK17-1 transcript. The detailed transcript analysis of miR531 and its predicted targets along with miR1429_5p and MPK17-1 were performed to gain deeper insights into their role (Table 1).

Experimental design and validation of predicted targets

The miRNA mediated regulation involves either target transcript degradation or translational repression.⁷ Therefore by principle, the increase in miRNA transcript expression would decrease the target transcript levels and vice-versa, displaying an inverse correlation between miRNA and corresponding transcript. Such observations of inverse transcript correlation were previously reported in *Arabidopsis* under diurnal conditions.¹² The same was applied to a few of the predicted miRNA: target pairs. The analysis were performed by qRT-PCR using ubiquitin as internal control. The rice plants were subjected to two abiotic stress conditions, namely drought and UV-B rays to study the inverse correlation between miRNA and its targets. Seven time points corresponding to 0h, 0.5h, 1h, 3h, 6h, 12h, and 24h were selected for harvesting stress treated samples. For positive control experiments, validated miRNA:target pairs – miR156a:SPL1 and miR168a:Ago1a reported in rice system were used.¹³

Table 1. Selected miRNA: target pairs for validation

Sl. No.	miRNAs	Targets (Gene IDs)	Target sequence	Free Energy	Source
1	miR1429_5p	MPK17-1 (LOC_Os06 g49430)	2260-UGCAUGGAUUAUGUAUUU	-26.7	PMRD
2	miR531	MPK3 (LOC_Os03 g17700)	571-ACAUGAUGACGGAGUACGUGGUCACCCGGUGG	-33.4	Microinspector
3	miR531	MPK6 (LOC_Os06 g06090)	0-AUGGACGCCGGGGCGCAGCCGUCGGACACGGGA	-36.6	Microinspector
4	miR531	MPK20-1 (LOC_Os01 g43910)	31-CAGAGGCGGAUUUUUACGAGUAUGGCGAU	-33.51	Microinspector
5	miR531	MKK10-2 (LOC_Os03 g12390)	889-AGAAGGCCGGCGCGCGUCGUCGGCGGAG	-46.2	Microinspector
6	miR531	MKKK3 (LOC_Os02 g32610)	25-CGAUGGUGUCUGCGGGCGGGCGGGCGGGCGGG	-40	Microinspector
7	miR531	MKKK4 (LOC_Os02 g12810)	207-GCUCCGUGGGCGGGCGGGCGGGAGCCGAG	-37.9	Microinspector
8	miR531	MKKK19 (LOC_Os02 g35010)	328-GCGGUCGCGGGCGGAGUCGUCUGCGGGCGGG	-38.8	Microinspector
9	miR531	MKKK23 (LOC_Os12 g40279)	26-GCGGUCUGGGCGGGCGGUCGGCCUGGGCUGG	-42.9	Microinspector
10	miR531	MKKK28 (LOC_Os03 g15570)	702-GAGGAGGAUAGCCAUCACGCCCGGGCGAG	-39.1	Microinspector
11	miR531	MKKK30 (LOC_Os02 g02780)	296-GCUGGAGGAGGCGCGCGGAUCCGGACCGGGCGG	-38.2	Microinspector
12	miR531	MKKK33 (LOC_Os02 g07790)	184-CGGUGGACAACAGCAGCGUUGGUCGGAACGAG	-32.1	Microinspector
13	miR531	MKKK38 (LOC_Os06 g45300)	179-CUUUGUUGGUGGUUCGGUUGCCAUGUCGGUGG	-31	Microinspector
14	miR531	MKKK45 (LOC_Os06 g43030)	208-UGCUGGACACGGCCAUGGGGCCUCCGGCGAG	-38.8	Microinspector
15	miR531	MKKK47 (LOC_Os07 g08750)	816-CUCGCCCGGGCGGGCGGGCGGGCGGGCGGAG	-46.1	Microinspector
16	miR531	MKKK52 (LOC_Os12 g06490)	15-GCGGAGGCGGAGGAGCAGCCCGGGACGAGGA	-42.2	Microinspector
17	miR531	MKKK57 (LOC_Os05 g46750)	143-GGGGCGGGCGGGCGGGCGGAGCUGCGGGCGGG	-43.1	Microinspector
18	miR531	MKKK58 (LOC_Os03 g39150)	601-AGUGUCUCCAAGGCGGGCACGUUCCGGUGG	-35.2	Microinspector
19	miR531	MKKK59 (LOC_Os12 g41260)	35-GGUGGAGAGAAGGCGGGCGGGCGGGCGGGUGG	-36.9	Microinspector
20	miR531	MKKK60 (LOC_Os03 g53410)	574-GCGUGGUGGUGGUGGAGUACCAGCACGGCGGG	-36.4	Microinspector
21	miR531	MKKK66 (LOC_Os10 g04010)	82-UGUCUCUGCGGGCGGACGACGCCUCCGGCGAG	-41.4	Microinspector
22	miR531	MKKK67 (LOC_Os10 g04000)	131-CGAGGGCGGGCGGAGCAGCAGCUCGGCGGAG	-44.3	Microinspector
23	miR531	MKKK68 (LOC_Os12 g30570)	409-CCGGCGGGCGGGCGGGCGGGCGGGCGGGCGGAG	-43.2	Microinspector
24	miR531	MKKK69 (LOC_Os05 g46760)	3-GCGGUGGGCGGGCGGGCGGGCGGGCGGGUGAG	-41.9	Microinspector
25	miR531	MKKK70 (LOC_Os01 g50410)	113-CGCCGGGGCGGGCGGGCGGGCGGGCGGGCGGAG	-45.6	Microinspector
26	miR531	MKKK73 (LOC_Os03 g18170)	102-GAGGCCAAGGGCGGGCGGGCGGGCGGGCGGAG	-42.5	Microinspector

Table 2. Correlation coefficients of miRNA: target pairs

Sl. No.	miRNA:Target	UV-B	Drought
1	miR156::SPL1	-0.528	-0.265
2	miR168::Ago1a	-0.144	-0.403
3	miR1429-5p::MPK17-1	-0.039	-0.453
4	miR531::MKKK3	-0.221	-0.422
5	miR531::MKKK4	-0.388	-0.360
6	miR531::MKKK19	0.058	-0.416
7	miR531::MKKK23	-0.244	-0.716
8	miR531::MKKK28	-0.418	-0.543
9	miR531::MKKK33	-0.018	-0.570
10	miR531::MKKK38	-0.049	-0.311
11	miR531::MKKK52	-0.253	0.227

The expression analysis of all the selected miRNAs and the target genes along with control pairs (miR156:SPL1 and miR168:Ago1a) were performed. The RQ values were determined by normalizing against ubiquitin expression. The graphs were plotted by selecting the miRNA with its corresponding target. Clearly, in the control pairs the transcript accumulation were inversely correlated between the miRNA and its targets (Supplementary Figure 1). The correlation coefficients calculated between the expression values of miRNA and its target were negative for the control pairs suggesting the inverse expression pattern. Considering the expression patterns and correlation coefficients of the control pairs in both drought and UV-B stress treatments, it was sought to identify the similar patterns of expression and correlation coefficients in predicted miRNA: target pairs. In nine out of 26 predicted miRNA: target pairs, transcript accumulation pattern between predicted miRNA: target pairs were similar to the control set suggesting their biological significance (Table 2). Eight of those pairs included miR531 – predicted by Microinspector, while one was miR1429_5p – predicted by PMRD (Fig. 1 and Fig. 2). The validated targets of miR531 included only the members of MAPKKK namely, MKKK3, MKKK4, MKKK19, MKKK23, MKKK28, MKKK33, MKKK38 and MKKK52.

The minimum free energy for hybridization for each of the miRNA-target pair was calculated by submitting microRNA sequence and the RNA sequence of the predicted target to the RNAhybrid server.¹⁷ The image generated, depicts the hybridization between miRNA (green) and its corresponding target sequence (red) (Fig. 3).

Discussion

The present work focuses on the identification of miRNAs that target the transcripts of MAP kinase cascade components in *Oryza sativa*. Two in-silico tools – Microinspector and psRNAtarget (PMRD) were used to predict miRNAs for 99 members of MAPK cascade component transcripts. The miRNAs

predicted by both the online tools did not overlap and were different. Nine out of 26 (~35%) miRNA:target pairs were validated as functional miRNA:target pairs and among them eight targets were MAPKKKs. This fact clearly emphasizes the importance and requirement of miRNA mediated post-transcriptional regulation in MAPK signaling cascade, especially of MAPKKKs. This is in complete agreement with the regulation of RAS (a MAPKKK) by a miRNA let-7 in *C. elegans*.¹⁰ These independent observations from animal and plant systems indicate the functional conservation of the signaling molecules and silencing pathways as well as their intricate networks of regulation in the two major kingdoms of life.

The post-transcriptional regulation of MAPKKK transcripts by miRNAs would only result in the regulation of the complete MAPK cascade downstream of that particular MAPKKK. For instance, OsMKKK3 is the ortholog of CTR1 in *Arabidopsis*, which negatively regulates MKK9–MPK3/6 cascade.¹⁴ Transcriptional inhibition or silencing of OsMKKK3 by miR531 can activate the downstream MKK9–MPK3/6 pathway leading to the stability of EIN3 transcription factor. Similarly, MKKK4 is the ortholog of EDR1 which physically interacts with MKK4/MKK5 and negatively regulates the plant innate immunity.¹⁵ Further work is necessary to determine the regulation of the MAPKKK transcripts is due to transcript cleavage or translational inhibition.

Materials and Methods

Sequence retrieval and identification of miRNAs targeting MAP kinase transcripts in rice

All the mature miRNA sequences of rice were downloaded from miRBase Release 17 (<http://www.mirbase.org/>)¹⁶ and all the rice

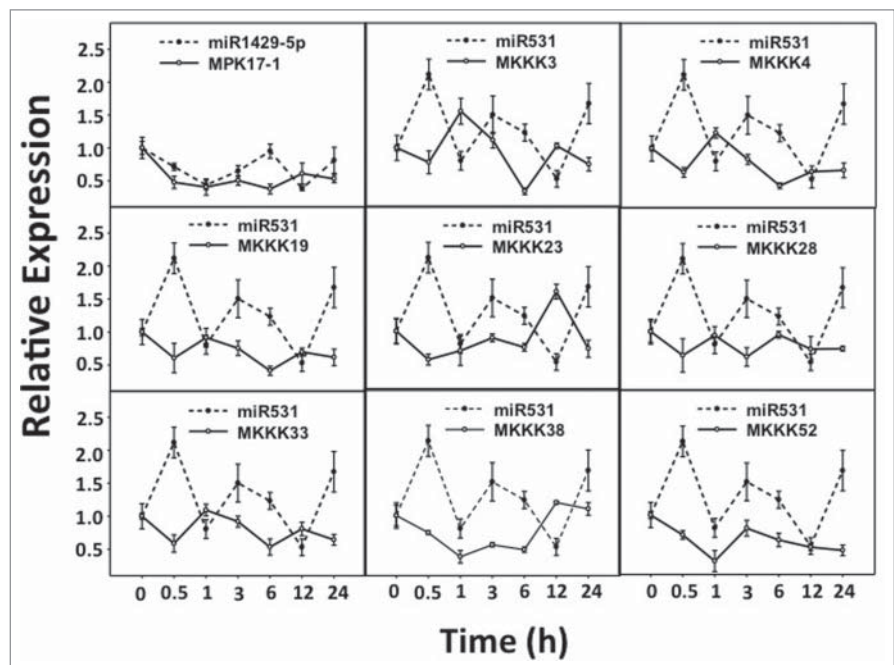


Figure 1. Expression patterns of predicted miRNA: target pairs under Drought stress. Relative expression levels of predicted miRNA: target pairs were analyzed under Drought stress for similar correlation patterns as the control set.

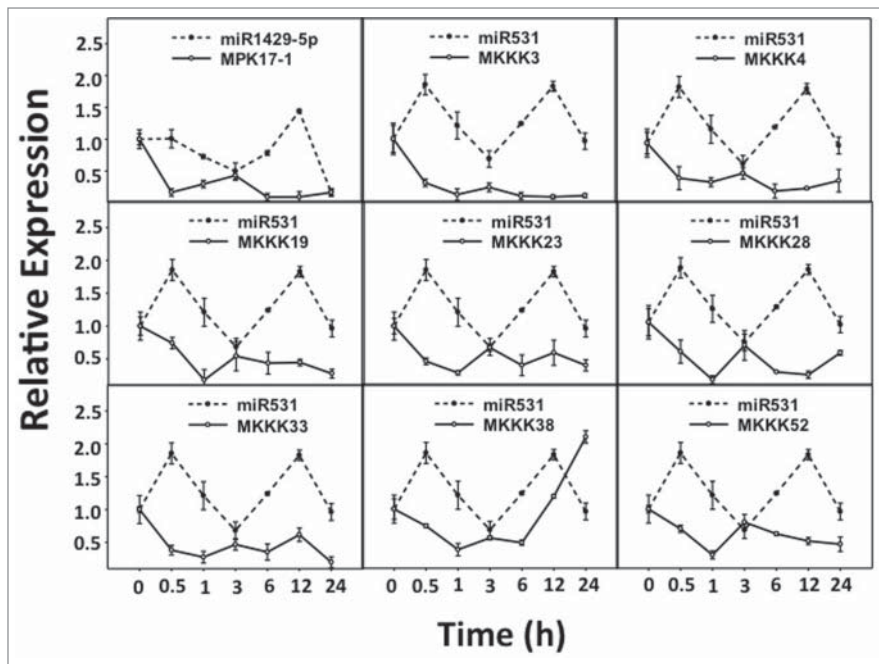


Figure 2. Expression patterns of predicted miRNA: target pairs under UV-B stress. Relative expression levels of predicted miRNA: target pairs were analyzed under UV-B stress for similar correlation patterns as the control set.

MAP kinase (16 MAPKs, 8 MAPKKs and 75 MAPKKKs) coding sequences were downloaded from Rice genome annotation project (<http://rice.plantbiology.msu.edu/>)¹⁷ database using gene IDs.²⁻⁴ Each of the MAP kinase coding sequence along with all the rice miRNA sequences were submitted to the Microinspector online tool (<http://bioinfo1.uni-plovdiv.bg/cgi-bin/microinspector/>).¹¹ The potential miRNAs targeting the given coding sequence, the position and the resultant target sequence of the CDS, and the free binding energy between the miRNA and the transcript were saved for each of the MAP kinases. Also, the search function of the PMRD (plant microRNA database) (<http://bioinformatics.cau.edu.cn/PMRD/>) was used to search the targeting miRNAs by querying all the MAP kinase gene IDs.¹⁸ This search function of PMRD relies on psRNATarget server.¹⁹

Plant growth conditions, maintenance and stress treatments

Rice (*Oryza sativa* L indica cultivar group var Pusa Basmati 1) was grown in

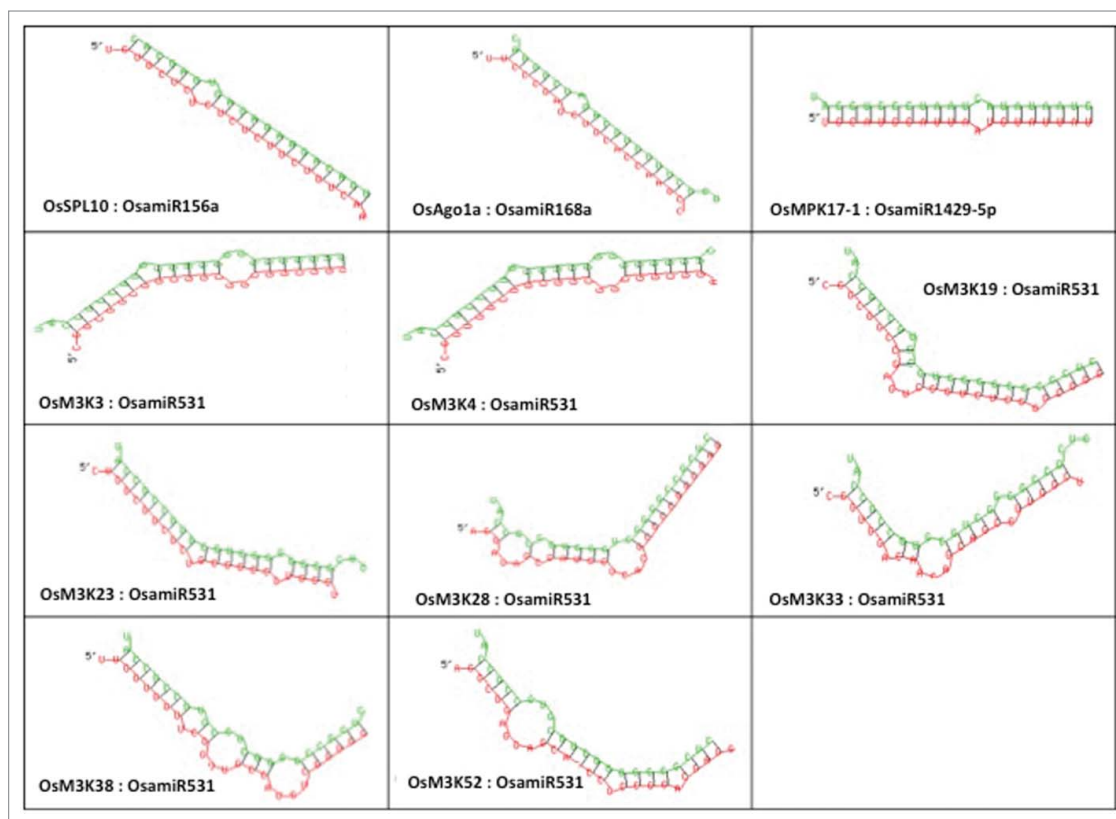


Figure 3. Representation of the interaction between the control miRNA: target pairs and validated miRNA: target pairs. The miRNA strand is represented in green and the target region in red.

growth chamber (SCILAB instrument, Taiwan) at 28 °C with 16 h light and 8 h dark period or grown in green house at NIPGR. Drought stress was induced by 15% polyethylene glycol-6000 to three week old rice wild type seedlings growing hydroponically with half strength Hoagland solution. UV-B treatment was given by exposing three week old rice wild type seedlings to UV-B tubes (Phillips, Netherland) for ten minutes under normal light conditions. Distance between UV tubes and plants were kept at ~20 cm. The plant samples were harvested at different time points mentioned in the legend of respective figures by snap freezing in liquid N₂ and stored in -80°C for further analysis.

RNA isolation, cDNA synthesis and qPCR analysis

RNA isolation was performed using TRI reagent® (Sigma) according to the manufacturer's instructions and cDNA was synthesized using RevertAid™ H Minus First strand cDNA synthesis kit (Fermentas). For miRNAs, stem-loop primers were designed as described by Varkonyi-Gasic et al.²⁰ and used in 50 nM final for each of the miRNAs reverse transcribed. Equal concentrations (100ng) of the total RNA was used for the reverse transcription along with oligo(dT)₁₈ and stem-loop primers. qPCR primers for all the MAP kinases were designed from the 3'-UTR regions using Primer Express (Applied Biosystems) software so as to ensure the uniqueness of the primers for each of the studied gene. All the qPCR study was performed on ViiA™ 7 (Applied Biosystems) platform using Power SYBR Green PCR master mix (Applied Biosystems). The concentrations of the

primers used were first standardized by running the standard curve protocol using successive five dilutions of a cDNA (1:1 till 1:625). Relative expression level of each gene was calculated using $\Delta\Delta C_T$ method and by normalizing against Ubiquitin as reference genes.²¹ The fold change represents the average value of two biological and independent replicates with three technical replicates each. All the primer sequences used are mentioned in the **Supplementary Table 1**.

RNA hybrid analysis

The minimum free energy for hybridization for each of the miRNA-target pair was calculated by submitting microRNA sequence and the RNA sequence of the predicted target to the RNAhybrid server.²² The image depicting the hybridization between miRNA and its target was also used for analysis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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