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Research Article

Expression Alteration of Candidate Rice MiRNAs in Response to Sheath Blight Disease

Soheila Talesh Sasani[1,2], Bahram M. Soltani[1*], Rahim Mehrabi[3], Hossein Samavatian[4], Fereidoun Padasht-Dehkaei[5]

[1] Department of Molecular Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran.
[2] Department of Biology, University of Guilan, Rasht, Iran
[3] Seed and Plant Improvement Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran
[4] Department of Plant Protection Research, Isfahan Agricultural and Natural Resources Research and Education Center, AREEO, Isfahan, Iran.
[5] Plant Protection Department, Rice Research Institute of Iran (RRII), Rasht, Iran

*Corresponding author: Bahram M. Soltani, Department of Molecular Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran. Tel: +98-2182884703; fax: +98-2182884717: E-mail: soltanib@modares.ac.ir

Background: MicroRNAs, as small non-coding RNAs, are recently reported to be involved in plant defense system against pathogens including fungi.

Objective: In this research, it was intended to investigate candidate susceptible rice (Oryza Sativa) Osa-miRNA expression alteration following the infection by *Rhizoctonia solani*.

Materials and Methods: To this aim, literature review suggested eight conserved plant miRNAs that are involved in other plant-pathogen interactions. Then, sixty days old rice plants (Hashemi, susceptible cultivar) were inoculated with R. *solani* and candidate miRNA expression alterations were investigated 2 hpi (hours post inoculation), 2 dpi (days post inoculation) and 6 dpi.

Results: RT-qPCR analysis suggested four subgroups of candidate miRNAs based on the time of their responses to the pathogenesis of *R. solani*. While Osa-miR-156 was early-responsive, Osa-miR159 was the last-responsive and Osa-miR167, Osa-miR171, Osa-miR408, and Osa-miR444 were late responsive to *R. solani* infection. *Osa-miR166* and *Osa-miR393* were non-responsive to this infection, compared to the mock-inoculated control group. Consistently, Os-SPL3 and Os-MADS known target genes were expressed in reverse correlation to Osa-miR156 and Osa-miR444, respectively.

Conclusions: From these data, it is suggested that both early (Osa-miR-156) and late (Osa-miR167, Osa-miR171, Osa-miR408, Osa-miR444) responsive miRNAs might be involved in *R. solani* infection in rice plants.

Keywords: miRNA, *Rhizoctonia solani,* Rice plant

1. Background

Rice is an important crop which provides major food for more than 50% of people worldwide. Sheath blight disease caused by *Rhizoctonia solani*, is one of the most destructive rice diseases resulting in an estimated yield loss of up to 50% (1). Micro-RNAs (miRNAs) are small non-coding RNAs, 18-27 nucleotides in length (2, 3), which control many biological and physiological processes in eukaryotes. Micro-RNAs play an important role in gene regulation at the post-transcriptional level. These small molecules have complementarity to their target genes and regulate them through mRNA degradation or translational repression (4, 5). With some differences, plant miRNA biogenesis is similar to that in animals (6). Plant miRNAs are involved in various biological processes including signal transduction, organ formation, developmental phase change, protein degradation and response to biotic and abiotic stresses (2, 7, 8). The first report about miRNAs involvement in plant disease resistance was shown in *Arabidopsis thaliana*. Navarro et al. (2006) showed that perception of bacterial flagellin flg 22 peptide

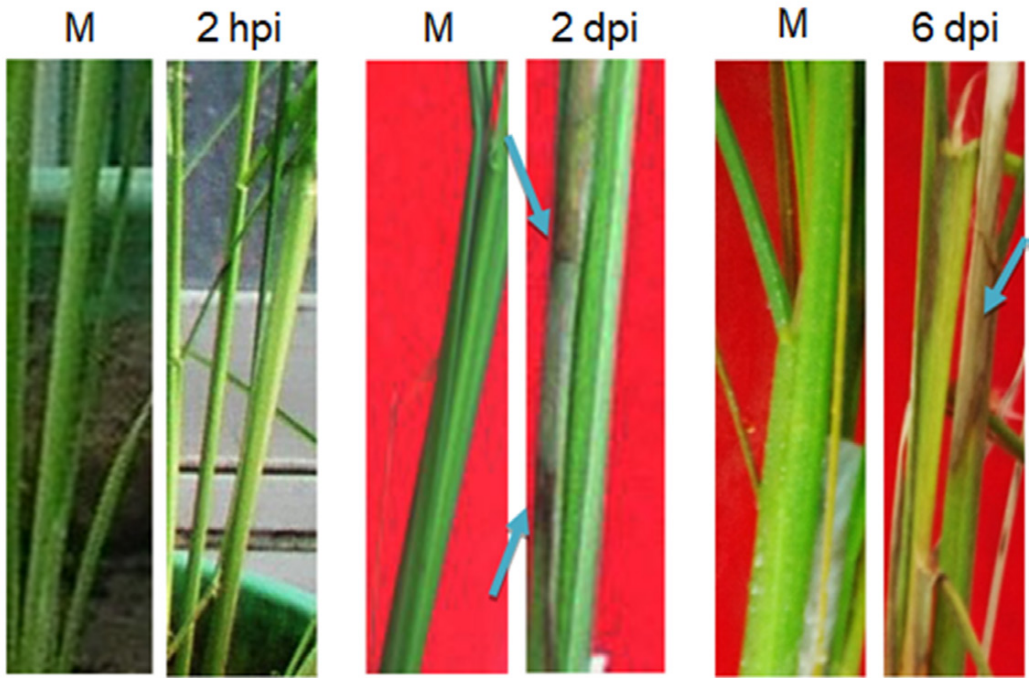

Figure 1. Pictures of rice plants inoculated by Rhizoctonia solani, the sheath blight causal agent. Disease symptoms (shown by arrows) were appeared 2 days after inoculation and are expanded to a maximum size at 6 dpi. Mock (M) shows rice sheaths treated with agar discs without pathogen.

led to the increase of At-miR393, down-regulation of auxin signaling and the increase of plant resistance (8, 9). The accumulation of the considerable number of miRNAs is expected to be affected through infection by a pathogen such as *R. solani*. The alterations in the accumulation of rice miRNAs during infection by *R. solani* have been recently reported. Many miRNAs are conserved among different species, which implies to their essential roles in many pivotal processes in plants. Conserved miRNAs have homologous target genes in different species which facilitates prediction of their targets. Here, we investigated the expression alteration of eight candidate conserved miRNAs and their target transcripts in rice plants which were challenged by *R. solani* fungus. At least five candidate miRNAs were responsive to the infection in susceptible rice plant.

2. Objective

In this research, it was intended to investigate candidate susceptible rice (Oryza Sativa) Osa-miRNA expression alteration following the infection by Rhizoctonia solani.

3. Materials and Methods

3.1. Plant Materials and Treatments

Seed of the rice (*Oryza sativa*) cultivar Hashemi which is susceptible to sheath blight disease, were let to germinate on moist filter papers and the resulted seedlings were transplanted into 13-cm pots containing clay loam soil. The pots were incubated in greenhouse (16 hrs light and 8 hrs dark cycle). A highly virulent pure culture of *R. solani* AG1-IA isolate G88 which has been isolated from Iran (10) was prepared by transferring of fungal sclerotia to 9-cm Petri dishes containing potato dextrose agar (PDA). Then, 60-day-old plants were inoculated with 5- mm agar discs of *R. solani* culture. The pots were transferred to greenhouse under 25-30 °C optimum temperature and 95% humidity conditions. As negative controls, some of the plants were inoculated using agar discs without pathogen. Samples of infected and mock-inoculated sheaths were prepared after 2 hpi (hours post inoculation), 2 dpi (days post inoculation) and 6dpi (**Fig. 1**).

3.2. RNA Extraction and cDNA Synthesis

Total RNA was extracted from infected and control plant samples using Trizol reagent (Ambion, USA) according to the manufacturer's protocol. Extracted RNAs were treated by using DNase I (Fermentas, USA) for removing any genomic DNA contamination. RNAs were polyadenylated using poly A polymerase. First-strand cDNA was synthesized using PrimeScriptTM 1st strand cDNA synthesis kit (Takara, Japan) and a mix of anchored Oligo-dT, in a 25 µL total reaction volume.

Table 1. Candidate miRNAs responsive to rice sheath blight disease

Osa-miRNAs	Location of the target site	Target gene
Osa-miR156	CDS & 3' UTR	SBP domain containing protein
Osa-miR159	CDS	MYB family transcription factors
Osa-miR166	CDS	START domin containing protein
Osa-miR167	CDS	Auxin response factor
Osa-miR171	CDS	SCARECROW gene regulation
Osa-miR393	CDS	Transport inhibitor response1 protein
Osa-miR408	3' UTR	Plastocyanin-like protein
Osa-miR444	CDS	MADS-box transcription factor

Table 2. List of primers used in this study

Primer	Sequence (5'-3')	Tm (°C)
osa-miR156	TGACAGAAGAGAGTGAGCAC	54
Osa-miR159	TTTGGATTGAAGGGAGCTC	52.3
Osa-miR166	TCGGACCAGGCTTCATTCC	57.2
Osa-miR167	TGAAGCTGCCAGCATGATCT	56.9
Osa-miR171	TGATTGAGCCGCGCCAATA	57.4
Osa-miR393	TCCAAAGGGATCGCATTGATC	55.2
Osa-miR408	CTGCACTGCCTCTTCCCTG	58.3
Osa-miR444	TTGCTGCCTCAAGCTTGC	56.7
Universal	AACTCAAGGTTCTTCCAGTCACG	60
tae-U6 (forward)	CGGGGACATCCGATAAAATTGG	62.1
tae-U6 (reverse)	TTGGACCATTTCTCGATTTGTG	58.4
SBP (forward)	AGCATGCTCTCTCTCTTCTGTCA	62.9
SBP (reverse)	CCAGGGTGAATCTGAGAACCT	61.3
MADS (forward)	CGGCCGCCTCTACGAGTAC	63.8
MADS (reverse)	TGCTGCTCATCCTTGGACTTG	61.3
Tae-actin (forward)	TGTTCCAGCCATCTCATGTTGG	62.1
Tae-actin (reverse)	TCATGCGATCAGCAATTCCAGG	62.1

3.3. Primer Designing for miRNAs and Detection of miRNA Target Genes

Based on the known sequence of plant conserved miRNAs, responsive to biotic and abiotic stresses, a total of eight miRNAs including Osa-miR156, Osa-miR159, Osa-miR166, Osa-miR167, Osa-miR171, Osa-miR393, Osa-miR408, and a monocot-specific miRNA, Osa-miR444 were selected from miRBase database (www.mirbase.org) and used for further analyses (**Table 1**). The sequences of these miRNAs were used as forward primers (with minor modifications at 3' and 5' ends) together with a universal reverse primer in qRT-PCR (**Table 2**). For selection of the miRNAs target genes miRBase (http://www.miRbase.org/) and TarBase (http://diana.cslab.ece.entua.gr/tarbase) online tools were employed

3.4. Quantitative Real-Time PCR Analysis

In order to study the expression profiles of selected miRNAs in response to rice sheath blight disease, RT-qPCR was performed using ABI (Applied Biosystems StepOne). Real-Time system was set up using CYBR Green I as follows: 94 °C for 15 min, 40 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 20 s and a final extension at 72 °C for 8 min. Quantitative RT-PCR data were analyzed using GraphPad Prism tool and each time point data of *R. solani* treated plants were compared to mock-inoculated plants data at the same time point using 2–ΔΔCT method, and were used in order to calculate the fold changes in the expression of the studied genes. Wheat U6 (tae-U6) gene which is well conserved in rice was used as reference gene. Polyacrylamide gel electrophoresis (PAGE) was performed to confirm qRT- PCR amplification products followed by cloning of the products in TA-pTG19 cloning vector and transformation in competent *Escherichia coli* (DH5α). The transformed colonies were selected and plasmid isolation was performed using plasmid extraction kit (GeneAll, Korea) followed by sequencing.

3.5. Designing Primers for Target Genes

SBP-Box transcription factor SPL3 is a direct upstream activator of LEAFY, FRUITFULL, and APETALA1 factors which are known to be involved in plant

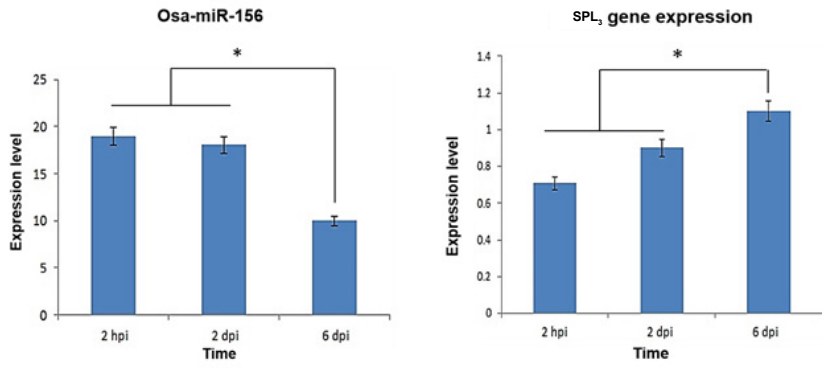

Figure 2. Real-Time PCR expression alteration of early responsive Osa-miR156 and its target gene, SPL3, in response to rice sheath blight disease, compared to untreated plants. This miRNA is upregulated as early as 2 hpi and last for at least 2dpi. Then, its relative expression is significantly decreased at 6dpi. Data normalization was performed by using wheat U6 and actin genes for Osa-miR156 and SPL3 expression, respectively. Results are the mean of three independent replicates.

development. SPL_3 transcription factor gene as a target for Osa-miR156, and, Os-MADS gene as a target for miR444 were used for primer design. The primers were designed using the software primer3 web (version 4.1.10) freely available online (http://primer3.ut.ee/) and were further analyzed using Oligo-analyzer software (http://euidtdna.com/calc/analyser).

3.6. Real-Time Analysis of Target Genes Expression Pattern

Real-time amplification of target genes was performed using an ABI (Applied Biosystems StepOne) system with CYBR Green master mix (BioFact, South Korea) as following thermal cycles: 94 °C for 15 min, 40 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 20 s and final extension at 72 °C for 8 min. Wheat actin gene that is conserved in rice was used as endogenous control.

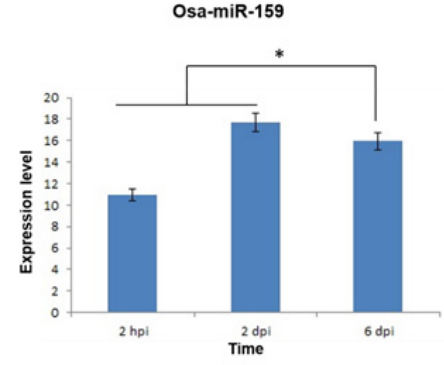

Figure 3. RT-qPCR expression alteration of mid-late responsive Osa-miR159, in response to the rice sheath blight disease, compared to untreated plants. This miRNA is upregulated as early as 2 dpi and last for at least 6dpi. Data normalization was performed by using wheat U6 for Osa-miR-156. Results are the mean of three independent replicates.

4. Results

4.1. Alteration of Candidate Osa-miRNAs Expression in Response to Sheath Blight Disease

In order to investigate the expression alteration of candidate rice miRNAs in response to sheath blight disease, rice plants were inoculated with *R. solani* (**Fig. 1**). Then, expression level alteration of some conserved rice miRNAs (Osa-miRNAs) was investigated 2 hpi, 2 dpi and 6 dpi in pathogen-infected or mock treated plants. RT-qPCR analysis suggested four subgroups of candidate miRNAs based on the time of their responses to the pathogenesis of *R. solani*.

4.2. Early-Responsive Osa-miR156

The expression of Osa-miR156 was significantly increased as early as 2 hpi in susceptible rice plants with *R. solani* and lasted for 2 days (**Fig. 2**). Because of the almost immediate response of Osa-miR156 to the infection, this miRNA was categorized as early-responsive to *R. solani* infection. On the other hand, the SPL3 gene has been reported as a target gene for Osa-miR156 (11). Therefore, SPL3 gene expression status was also investigated in the same samples and a negative correlation of expression was deduced between them (**Fig. 2**). At this time point, the preliminary symptoms of disease were detectible on the infected leaves (**Fig. 1**).

4.3. Late-Responsive miRNAs

RT-qPCR results indicated that Osa-miR159 expression level was increased at 2 dpi and lasted until 6 dpi (**Fig. 3**). Therefore, Osa-miR159 was categorized as a late responsive miRNA to *R. solani* infection. Compared

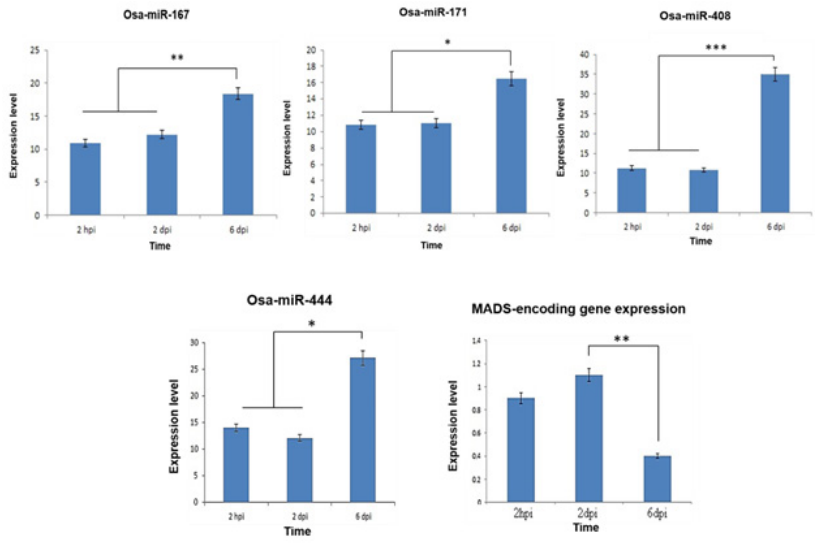

Figure 4. RT-qPCR results show synchronized expression alterations of Osa-miR167, Osa-miR171, Osa-miR408, and Osa-miR444 in response to rice sheath blight disease, compared to untreated plants. The expression of an OsMADS encoding gene as a known target gene for Osa-miR444 has also been investigated in the same samples, showing a negative correlation with Osa-miR444 expression status. Wheat U6 gene was used for data normalization. Results are the mean of three independent replicates.

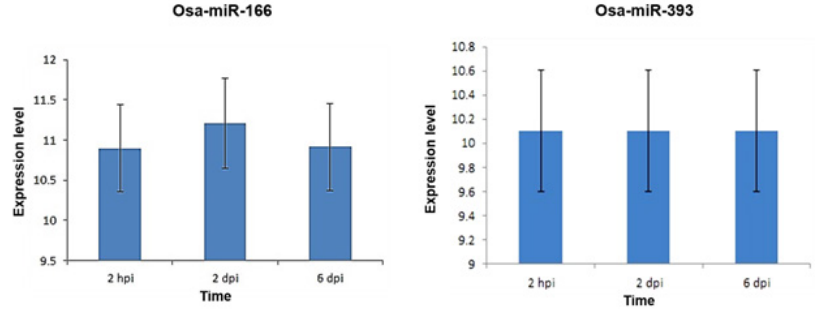

Figure 5. Depicts non-significant expression alteration of Osa-miR393 and Osa-miR166, in response to the rice sheath blight disease using RT-qPCR, compared to untreated plants. Results are the mean of three independent replicates

to other time points and control group, the level of Osa-miR167, Osa-miR171, Osa-miR408, and Osa-miR444 expression was increased about 6 dpi (**Fig. 4**). These miRNAs were categorized as the miRNAs late-responsive to the infection by *R. solani*. Transcripts of OsMADS gene are known as targets for Osa-miR444, therefore OsMADS expression status was investigated in the same samples. While Osa-miR444 expression level was increased 6 dpi, Osa-MADS expression level was decreased following the disease development (**Fig. 4**). At this time point, the strongest symptoms of disease were detectable (**Fig. 1**).

4.4. Non-Responsive miRNAs

RT-qPCR results indicated that Osa-miR166 and Osa-miR393 did not exhibit any significant expression alterations in the infected rice compared to the control plants (**Fig. 5**).

5. Discussion

Some of the rice lines such as Jasmine 85 are reported to be partially resistant to sheath blight disease (12, 13). However, no complete resistant rice variety has been reported yet (13). MicroRNAs as a group of small non-coding RNAs play important roles in the biological processes including plant-pathogen interactions. Conservation of plant miRNAs emphasize their function and here, it was intended to analyze the expression status of some conserved candidate plant miRNAs following the challenge of rice with sheath blight disease. Of course, we had in mind that being conserved and being involved in response to other disease resistances, do not

guarantee that candidate miRNAs are associated with resistance to the sheath blight disease. Also, response to a stimulus is different than the expression alteration upon a stimulus. Never the less, miR156, miR159, miR166, miR167, miR171, miR393 and miR408 are conserved between wheat and rice (14). Also, some of these miRNAs are conserved in maize, Arabidopsis, and Sorghum. Therefore, expression status of these candidate miRNAs was investigated in response to the sheath blight disease in Hashemi rice variety, regarded as a susceptible cultivar.

5.1. Early-Responsive Osa-miR156

The expression of Osa-miR156 was significantly increased as early as 2 hpi in susceptible rice plants challenged with *R. solani* and lasted for 2 days (**Fig. 2**). Consistently, Tae-miR156 expression level has been reported to be increased following the viral infection of arabidopsis (15), rust infection (16) and heat stress (17) of wheat plants. However, its expression level has been down-regulated following the infection of at least two wheat genotypes by *Blumeria graminis* (17). At-MiR156 is involved in Arabidopsis development through regulation of 16 members of SPL (squam promoter binding protein-like) gene family. SPLs are known to be involved in plant developmental phase change (11, 18) and also in plant response to stressors like copper and fungal toxins (19). There are 19 SPL genes in rice, 11 of them have miRNA Responsive Element (MRE) recognized by Osa-miR156 (5, 20). Particularly, SPL3 gene has been reported as a target gene for Osa-miR156 (11). Here in the present article, the expression pattern of SPL3 gene and Osa-miR156 was compared in the infected rice samples and a negative correlation was found between them (**Fig. 2**). Such a negative correlation has been reported between this miRNA and its target genes in powdery mildew-infected wheat cultivars (7, 17). Overall, our findings suggest that Osa-miR156 may affect rice response to sheath blight disease probably through the alteration of *SPL3* gene expression level. This miRNA may be considered as an early marker for this infection in rice.

5.2. Mid-Late Responsive Osa-miR159

The expression level of Osa-miR159 was increased 2 dpi and lasted until 6 dpi (**Fig. 3**). These results are consistent with the earlier findings showing that the expression of Osa-miR159 was increased in wheat upon yellow rust disease infection (16) and drought stress in rice (3). The miR159 has an important role in plant development by targeting MYB transcription factors which affect plant responses to different stresses (21, 22). MiR159 has other non-conserved targets including Cu/Zn SOD genes (4) encoding pathogenesis-related proteins peroxidase (POD) and cytokinin oxidase (CKX) (8). These target genes have important roles in Populus tricocarpa resistance to fungal diseases and in increased arabidopsis resistance to club-root disease caused by Plasmodiophora brassicae (8). Overall, Osa-miR159 may affect rice response to sheath blight.

5.3. Late Responsive Osa-miRNAs

Real-time analysis indicated that Osa-miR167, Osa-miR171, Osa-miR408, and Osa-miR444 expression levels have been increased about 6 dpi, compared to other time points and control plants (**Fig. 4**). Our finding is in consistency with the up-regulation of miR167 during disease progress and salt, drought and powdery mildew disease stress in Arabidopsis and wheat, respectively (17, 23). However, Osa-miR167 expression level has been decreased following drought stress (24) and ABA treatment in rice and in arabidopsis plants infected by the nematode, *Heterodera schachyii* (7). MiR171 expression has been reported to be increased in rice and arabidopsis under cold and salinity stresses (7), whereas it has been reported to be decreased under drought and oxidative stresses (25). Both Osa-miR167 and Osa-miR171 are known to regulate auxin signaling through Auxin Response Factors (ARFs) targeting. Target genes of miR408 are known to be involved in programmed cell death, pathogen infection and defense responses (8, 26). Consistently, miR408 expression has been increased in response to salinity, cold and oxidative stress in Arabidopsis. The expression of Osa-miR444 as a monocot-specific miRNA (14) was investigated along with its known target, OsMADS gene. While Osa-miR-444 expression level was increased 6 dpi, Osa-MADS expression level was decreased following the disease progression (**Fig. 4**). Consistently, *Blumeria graminis* infection in wheat susceptible cultivar (JD8), as well as cold stress in rice, have all led to the increased expression level of miR444 (7).

5.4. Expression Alterations of Osa-miR166 and Osa-miR393

Osa-miR166 and Osa-miR393 had no significant expression alterations in the infected rice compared to the control plants (**Fig. 5**). However, miR166 is reported to be down-regulated 8 h after drought stress in *Triticum dicoccoides* (2) and in rice (3). MiR393 is known to be involved in plant basal defense against pathogens. While, its overexpression in *Arabidopsis thaliana* increases susceptibility to *Alternaria brassicicola*, enhances resistance to *Hyaloperonospora*

due to its effects on auxin receptors. Overall, it seems that unlike their common expression alteration in other plant pathosystems, Osa-miR166 and Osa-miR393 are not directly involved in rice *R. solani* interaction.

6. Conclusion

To get insight into the possible role of rice miRNAs in response to fungal sheath blight disease the expression level of eight conserved miRNAs and some of their target genes were studied in the present study. These miRNAs were classified into; early responsive, mid-late responsive, late responsive and no responsive miRNAs. From these data, it is suggested that early (Osa-miR-156) and late (Osa-miR167, Osa-miR171, Osa-miR408, Osa-miR444) responsive miRNAs may be involved in rice plant response to the infection by *R. solani*. It remains to be tested if the expression pattern of these miRNAs are different in rice plants with different degrees of resistance to disease and are dose dependent on the infection. Also, it remains to be tested if the expression alteration of these candidate miRNAs is active response initiated by plants or a passive consequence due to the stimulus.

Acknowledgments

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