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Expression Analysis of Genes and MicroRNAs in the Rice Cultivars during Infection by *Xanthomonas oryzae p.v. oryzae*

Anjum N, Methre R, Boghireddy S, Laha GS, Sundaram RM and Mangrauthia SK*

ICAR-Indian Institute of Rice Research, Hyderabad 500030. India
*Corresponding author (skmdrr@gmail.com)

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Abstract

Bacterial blight (BB) disease of rice, caused by *Xanthomonas oryzae p.v. oryzae* (*Xoo*) results in enormous losses in grain yield world-wide and India. In this study two popular and high yielding near isogenic lines of *Oryza sativa* indica –Samba Mahsuri (SM) and Improved Samba Mahsuri (ISM) which differ only in their resistance against BB (driven by three resistant genes *Xa21, xa13* and *xa5*) were used to analyse the expression of genes and microRNA s after infection by Xoo. Five miRNAs (miRs 162, 167, 168, 169 and 398) and five genes (ARF, NBS-LRR, NTF-Y, SOD and CWIP) were chosen for expression analysis using Real Time PCR to understand the differential gene expression and regulation of these two genotypes after infection by the pathogen. The genes and miRNAs were selected based on their conserved nature and diverse functions in plant growth and metabolism. All the selected genes showed similar expression pattern in both- SM and ISM suggesting that these NILs have similar genetic architecture. However, expression of miRNAs was differentially regulated in these genotypes. Specifically, miR162 and miR398 were differentially expressed. miR162 was significantly down-regulated in ISM while it showed increased expression in SM. miR168 expression was also drastically reduced in ISM after BB infection. miR162 and miR168 regulate expression of Dicer and Argonaute proteins, respectively, which in turn control biogenesis of miRNAs. This study shows that introgression of BB resistance genes into SM affected gene regulation pathways mediated by miRNAs. The study suggests the important role of miRNAs in BB resistance mediated by *Xa21, xa13* and *xa5* genes. It would be interesting to further decipher the function of miRNAs in BB resistance.1

Key words: miRNA; *Oryza sativa; Xanothomonas*; mRNA; gene regulation

Introduction

Rice is most important food crop in India and covers maximum area under cultivation. With the increase in population, food security and social stability of India depends to a significant extent on genetic improvement of rice cultivars and fine-tuning of production technologies to meet the growing challenges. In order to enhance rice production, it is important to increase its yield by deploying conventional and molecular tools. In parallel, efforts have to be made to manage abiotic and biotic stresses which are major constraints in attaining higher rice productivity. In particular, the introduction of high yielding, fertilizer responsive rice during and post green-revolution era has increased the incidences of several biotic stresses. Among the rice diseases- blast, bacterial blight, sheath blight, false smut, and rice tungro virus disease cause severe loss of grain yield and quality. Bacterial blight (BB) caused by *Xanthomonas oryzae p.v. oryzae* (*Xoo,* a gram negative bacterium) can reduce rice yields up to 50% in favourable environments like irrigated and rain-fed lowlands (Priyadarishini and Gnanamanickam, 1999). In India, the BB disease is prevalent in most of the rice growing states.

At ICAR-Indian Institute of Rice Research (ICAR-IIRR) Hyderabad, the rice cultivar-Improved Samba Mahsuri (ISM) possessing high yield, fine-grain type and high level of resistance against bacterial blight (conferred by the resistance genes, *Xa21, xa13* and *xa5*) was developed in collaboration with CSIR-Centre for Cellular and Molecular Biology (CCMB), Sundaram *et al.,* (2008). This variety is a near isogenic line (NIL) derived from the elite rice variety, Samba Mahsuri (BPT5204) through deployment of molecular marker-assisted backcross breeding. It is noteworthy to indicate that Improved Samba Mahsuri possesses similar genetic composition to that of Samba Mahsuri except for the presence of BB resistance genes and the same is reflected in terms of plant phenotype. At molecular level, though both the genotypes have similar

genomic architecture, there is possibility that introduction of BB resistance genes might be responsible for modulation of expression and regulation of a few genes including micro RNAs (miRNAs) Recent studies showed that beside the protein coding genes, miRNAs also play a major role in stress response of plants by regulating number of transcription factors and metabolic genes, Sunkar *et al.,* (2007); Sailaja *et al.,* (2014); Agarwal *et al.,* (2015); Mangrauthia *et al.,* (2017). MicroRNAs are endogenous single-stranded 20-22 nt small RNAs regulating gene expression in plants and animals, Tang *et al.,* (2003); Bartel *et al.,* (2004). These small non-coding RNAs have also been demonstrated to regulate host defence against pathogens through transcriptional and post-transcriptional gene-silencing mechanisms. miRNAs have been reported to act as integral regulatory components of plant defence machinery against bacteria, fungi, and viruses. The present study was aimed to analyse the expression of set of known genes and miRNAs associated with defence response in Improved Samba Mahsuri vis-a-vis Samba Mahsuri under uninoculated and BB infection conditions to understand the differential gene regulation of these genes/miRNAs in the two rice varieties.

Materials and methods

Two rice varieties-Improved Samba Mahsuri (ISM, resistant to BB) and Samba Mahsuri (SM, susceptible to BB) were used as experimental materials. Seeds were surface sterilized in 0.1% $HgCl_2$ solution for 1 min followed by rinsing 3-4 times with distilled water. Treated seeds were germinated in a tray containing pulverised soil. After 20 days of germination, seedlings were transplanted into big pots. They were allowed to grow for 40 days under normal weather conditions. The BB pathogen, *i.e.*, *Xoo* (strain IX-020) was cultured in modified Walkimotos medium for three days and bacterial inoculums (10^8 cfu/ml) was prepared by suspending 3-days old bacterial growth in sterile distilled water. Sixty days old rice plants were inoculated with bacteria by following standard protocol used in AICRIP (All India Co-ordinated Rice Improvement Project) for screening of rice genotypes for BB resistance, Kauffman *et al.,* (1973). Plants were cut approximately 4 cm from the tip of fully expanded leaves with scissor dipped in a bacterial suspension. Among the two batches (eight plants/batch) of each variety grown, only one batch was inoculated and considered as test samples. Comparison of BB infected plants of SM and ISM is (Figure 1). The other batch was not inoculated by bacteria but was wounded by cutting the leaves with sterile scissors and was considered as control samples.

Leaves (10 days after inoculation) of control and *Xoo* infected plants of ISM and SM were used for isolating the total RNA and small RNA by using mirVana™ miRNA Isolation Kit (Ambion). The kit facilitates the extraction of small RNA and large RNAs (mRNA, rRNA) from same preparation. The total RNA was used for expression analysis of genes while small RNA was isolated for expression analysis of miRNAs. RNA was treated with DNase to remove contamination of DNA. Different procedures were followed for cDNA synthesis of genes and miRNAs. The cDNA for genes was synthesized by using oligo-dT primer and Improm-II reverse transcription system, as specified by manufacturer (Promega). miRNAs are not polyadenylated in nature. Therefore, addition of poly (A) tail to small RNAs was done by poly(A) polymerase and cDNA synthesis was done as per the protocol mentioned in miScript II RT Kit (Qiagen). Mature miRNA-specific PCR forward primers were designed based on miRNA sequences downloaded from miRBase (Table 1).

Table 1. List of forward primer sequences of miRNAs used in this study. The reverse primer was based on universal sequence tag provided in real time PCR kit.

Sl.No.	miRNAs	Sequence (5'-3')
1	162	TCGATAAACCTCTGCATC
2	168	TCGCTTGGTGCAGATCGG
3	167	TGAAGCTGCCAGCATGAT
4	169	CAGCCAAGGATGACTTGC
5	398	TGTGTTCTCAGGTCACCC
6	SnU6	CGATAAAATTGGAACGATACAGA

Reverse primer was based on universal tag sequence provided in kit. Gene specific forward and reverse primers used in this study (Table 2). U6 snRNA was chosen as an internal control, Ding *et al.,* (2011) for miRNA expression analysis while *OsActin1*, Lee *et al.,* (2011) was used as internal control for quantification of genes expression.

Table 2. The list of forward and reverse primers used for expression analysis of genes through real time PCR.

Sl. No.	Target gene	Accession No.	Primers
1	ARF	AK243230	Forward: CCGAGATGTTCTGCATCGACA
			Reverse: ATGGTAAGCCTCCTCGCCAAC
2	NTY	AK069348	Forward: TTCGTTCTATGGTGGTGCTGT
			Reverse: TTCGGTGGCTGGTTCTATTGG
3	SOD	AK110251.1	Forward: CCAGTGAACACAATGCTGGA
			Reverse: CTTGAGCCAGACGAACAACA
4	NBSLRR	Os07g29820.1	Forward: GCGTGCTCATGCGGAGATAA
			Reverse: AGCTGCGAGCAATGTGAACAA
5	CWIP	AK242237	Forward: AAGAAAAGCTCGTGGATGCAGA
			Reverse: AGCAGCCGCCTCATGGCAAT
6	Actin	AK100267	Forward: CAGCCACACTGTCCCCATCTA
			Reverse: AGCAAGGTCGAGACGAAGGA

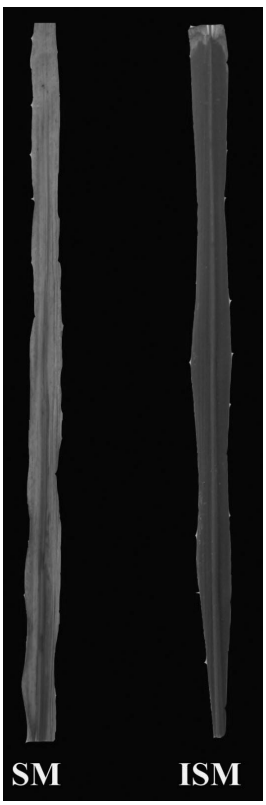

Figure 1. Phenotypic comparison of rice cultivars Samba Mahsuri (BPT5204) and Improved Samba Mahsuri (RP Bio-226) after infection with *Xanthomonas oryzae p.v. Oryzae.*

cDNA was treated with RNase and normalized for equal concentration. Quantitative PCR of miRNAs was performed using miScript SYBR Green PCR Kit (Qiagen) while SYBR Premix Ex-Taq (Takara) was used for quantification of genes. The 25 µL reaction volume consisting of 2.5 µL of normalized cDNA, 10 × miscript universal primer, 10 × miRNA specific forward primers and 12.5 µL of 2× quantitative SYBR green PCR master mix was prepared. Reaction mix for quantification of genes and amplification conditions were followed as described previously, Sailaja *et al.,* (2014). qRT-PCR reactions were performed in 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Three biological replications were run in duplicate and the melting curves were generated using Dissociation Curves Software (Applied Biosystems). ΔΔCt method was used for calculation of fold change expression and analyzed as described in Mangrauthia *et al.,* (2017).

Results and discussion

In the present study, two rice cultivars namely, SM and ISM were used for studying the expression of selected genes and miRNAs after infection by the bacterial blight pathogen, *Xoo*. The objective of this study was to understand the changes in the expression and regulation of selected genes and miRNAs in the two rice varieties, which are NILs differing only for BB susceptibility (*i.e.* Samba Mahsuri) or resistance (*i.e.* Improved Samba Mahsuri). Since both Improved Samba Mahsuri (ISM) and Samba Mahsuri (SM) possess similar genetic composition except for the presence of the BB resistance genes (*Xa21, xa13* and *xa5*) in ISM, it was planned to analyse the expression of a selected set of genes and their corresponding miRNAs which might have altered due to introduction of BB resistance genes. To understand the expression pattern of miRNAs and genes, five representative miRNAs chosen were - miR162, miR168, miR167, miR169, miR398. These are conserved miRNAs in plants and have been studied extensively for their various roles in plant growth and development and disease response (Kidner and Martienssen 2004; Zhang *et al.,* (2006). It is important to note that miR162 and miR168

are involved in regulation of biogenesis of microRNAs by regulating the expression of Dicer and Argonaute proteins, respectively, Xie *et al.,* (2003); Kidner and Martienssen, 2004; Vaucheret *et al.,* (2004). Important role of miRs 169, 398, and 167 has been reported in various biotic and abiotic stresses, Mangrauthia *et al.,* (2013); Sailaja *et al.,* (2014); Li *et al.,* (2017). Recently, Osa-miR169 was shown to regulate rice immunity against rice blast disease caused by *Magnaporthe oryzae,* Li *et al.,* (2017). miR398b was reported as negative regulator of pathogen-associated molecular patterns (PAMP)-induced callose deposition and disease resistance in Arabidopsis, Li *et al.,* (2010). The same study demonstrated the induced expression of miR167 by flg22, a well-studied PAMP. In another such report, miR167 was suggested to regulate disease resistance by fine-tuning plant hormone networks, while miR398 regulate the slicing

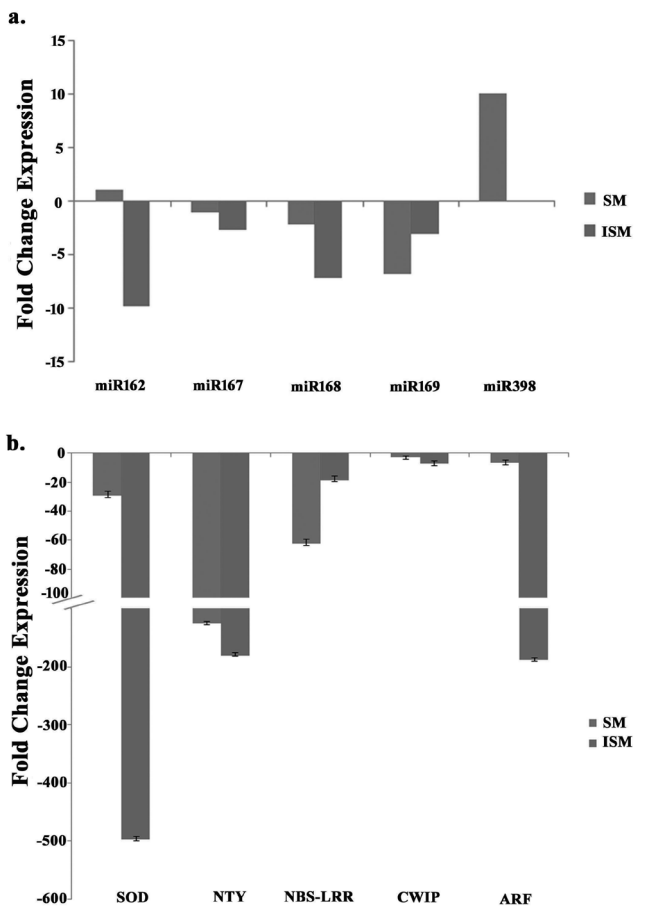

Figure 2. (a) Expression analysis of microRNAs. The Y-axis shows the fold change expression of miRNAs in BB infected Samba Mahsuri and Improved Samba Mahsuri rice cultivars in comparison with their respective control. (b) Expression analysis of genes. The Y-axis shows the fold change expression of genes in BB infected Samba Mahsuri and Improved Samba Mahsuri rice cultivars in comparison with their respective control. Bars represents the mean± SE of three biological replicates.

of genes inhibiting plant immunity, Huang *et al.,* (2016). Among the 5 miRNAs studied here, two miRNAs namely miR162 and 398 showed differential expression between SM and ISM after BB infection. These two miRs were up-regulated in SM, specifically miR398, which showed >6.0 fold upregulation. miR162 was significantly down-regulated in ISM while miR398 did not show expression. Expression of miR167 and 168 was decreased in both the genotypes, however, ISM showed high degree of down-regulation of the miRNAs as compared to SM (Figure 2a). It should be noted that miRNAs regulate the expression of their target genes negatively. Hence, down-regulation of miRNAs indicates up-regulation of their target genes.

MicroRNAs have been demonstrated to regulate host defence against plant pathogens through transcription and post-transcriptional gene-silencing mechanisms. A recent report suggested that several miRNA families act as regulators of plant immunity by regulating the expression of nucleotide-binding leucine-rich-repeat (NB-LRR) type resistance (R) genes of plant disease (Baldrich and San Segundo, 2016). Two miRNAs, nta-miR6019 and nta-miR6020 were shown to guide sequence-specific cleavage of transcripts of the TIR-NB-LRR immune receptor which confers resistance to tobacco mosaic virus. MicroRNAs are suggested as key players associated with fine-tuning of regulation of R-gene expression and reported to play a crucial role in disease resistance and plant fitness against diseases. One of the rice miRNA-osa-miR7695 was shown to negatively regulate the transcription of OsNramp6 (natural resistance-associated macrophage protein 6), and the over-expression of the miRNA enhanced resistance against pathogen infection in rice plants, Campo *et al.,* (2013). Another evidence of involvement of miRNAs in host-pathogen interaction emerged from the fact that Argonaute1, a key protein involved in miRNA biogenesis, is required for a number of PTI responses including PAMP-induced callose deposition, gene expression, and seedling growth inhibition, Li *et al.,* (2010). Though, extensive study has been done to identify genes associated with host-pathogen interaction, and specifically the genes associated with resistance pathways, very few attempts have been made to decipher the gene regulation mechanisms primarily mediated by microRNAs.

Representative genes selected for expression analysis in this study were auxin response factor (ARF), nuclear transcription factor-Y (NTY), nucleotide-binding site leucine-rich repeat (NBS-LRR), super-oxide dismutase (SOD) and cell wall integrity protein (CWIP). Expression analysis of these genes suggested down-regulation of all genes in both the genotypes. Therefore, SM and ISM

showed similar expression pattern of genes, though the level of expression varied significantly between the two varieties (Figure 2b). It can be expected to observe similar expression pattern with respect to these genes in both the genotypes as they represent similar genetic composition and such it can be hypothesized that these genes may not be associated with disease resistance mediated by *Xa21, xa13* and *xa5.* Even though introduction of BB resistance genes into SM through marker assisted selection did not affect the expression pattern of five representative genes, expression of miRNAs was found to be differentially regulated in both the genotypes, suggesting that gene regulation machinery has been altered due to introduction of BB resistance genes. It would be interesting to study that how introduction of *Xa21, xa13* and *xa5* affected the expression of miRNAs in ISM by studying NILs of Samba Mahsuri possessing only *Xa21* or *xa13* or *xa5* vis-à-vis Samba Mahsuri.

Conclusion

The role of miRNAs in BB resistance in rice has not been demonstrated yet, though the first resistance-related miRNA was reported in Arabidopsis infected with bacteria (*Pseudomonas syringae*), Navarro *et al.,* (2006). This study provides first base line information to further investigate the role of miRNAs in BB resistance driven by 'R' genes. Also, it would be important to further extend this study at genome-wide scale to decipher the role of miRNAs in Rice-Xoo interaction and regulation of resistance mechanism.

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