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Expression dynamics of *Glycine max* (L.) Merrill microRNAs (miRNAs) and their targets during *Mungbean yellow mosaic India virus* (MYMIV) infection

Shunmugiah V. Ramesh [a, *, 1], Bhagat S. Chouhan [b, 1], Gaurav Kumar [c], Shelly Praveen [c], Suresh Chand [b]

[a] Crop Improvement Section, ICAR- Indian Institute of Soybean Research (ICAR-IISR), Indore, Madhya Pradesh 452001, India
[b] School of Life Sciences, Devi Ahilya Vishwavidhyalaya (DAVV), Indore, Madhya Pradesh 452001, India
[c] Division of Biochemistry, ICAR-Indian Agricultural Research Institute (ICAR-IARI), New Delhi, India

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ABSTRACT

MicroRNAs (miRNAs) are class of small non coding RNAs (sncRNAs) that modulate gene regulatory mechanisms including conferring antiviral resistance in plants. Expressional changes of selected conserved miRNAs and putatively antiviral miRNAs were analyzed in two soybean genotypes (JS335 and UPSM534) with contrasting disease reaction trait against *Mungbean yellow mosaic India virus* (MYMIV) family *Geminiviridae*. Expression profiling of conserved miRNAs and corresponding target mRNAs in both the soybean genotypes suggests the role of Argonaute homeostasis in virus resistance along with the gene regulatory changes in hormonal signaling pathways. Putative antiviral miRNAs displayed upregulation upon MYMIV infection in both the soybean genotypes. Further, experimental evidence prove that soybean derived miRNA potentially target and direct cleavage of MYMIV mRNA encoding movement protein (BC1). Thus, this work provides molecular insights regarding changes in expression of miRNAs during MYMIV infection and improves deeper understanding of role of soybean miRNAs in MYMIV resistance. To the best of our knowledge this is the first report of soybean derived miRNAs exhibiting potential antiviral resistance during begomovirus infection.

1. Introduction

A gamut of plant derived small non-coding RNAs (sncRNAs) have been characterized as crucial component of gene regulatory mechanisms because of their diverse roles ranging from gene expression to defence against plant pathogenic viruses [1–3]. Among them, micro RNAs (miRNAs) are characteristically 21-24 nt small RNAs that modify gene expression at the post-transcriptional stage either by cleaving or by causing translational repression of the cognate target mRNAs [4–6]. miRNA genes have specific promoter sequences hence are transcribed by RNA pol II to generate primary miRNA transcript (pri-miRNA) [7]. The concerted activity of RNAse III-like proteins such as HYL/SER1 and DCL-1, followed by methylation and poly-uridylation of miRNAs leads to formation of mature duplex miRNAs from pri-miRNA [8,9]. Exponential growth in number of annotated miRNAs from diverse plant species and tissues has been possible due to deep sequencing of small RNA transcriptomes through cost-effective next generation sequencing (NGS) technologies, bioinformatic tools, and cloning approaches [10,11]. Plant miRNAs play decisive role in various processes such as growth and development of an organism including leaf morphogenesis [12], flower development [13], embryogenesis [14], preservation of genome integrity, and in response to biotic and abiotic stresses, etc [15]. Most importantly, majority of the miRNAs amend the expression pattern of transcriptional factors (TFs) hence are considered to be master regulators of key biological pathways in plants [5,16]. Plant miRNA families have been classified in to conserved and non-conserved miRNAs based on their conservation and diversification during the process of evolution [17,18]. Conserved miRNAs display sequence homology and also feature analogous target mRNA characteristics [19].

miRNAs have not only been shown to modulate gene expression related to plant developmental processes but also endow plants

* Corresponding author.
E-mail address: ramesh.sv@icar.gov.in (S.V. Ramesh).
[1] Authors contributed equally to this work.

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with molecular adaptive response to viral infections [20,21]. During virus infections, developmental abnormalities and virus induced symptoms are attributed to disturbed plant miRNA pathway [20–22]. *Arabidopsis*, infected by Tobacco mosaic virus Cg (TMV-Cg) or Oilseed rape mosaic virus (ORMV) exhibited elevated activity of miR164a promoter leading to over-expression of mature miR164a and simultaneous reduction of target transcript CUC1 [23]. This study further demonstrated that altered miRNA activity during virus infection possess a transcriptional gene silencing component as well [23]. The role of miRNA168 and its target mRNA Argonaute 1 in conferring host resistance during virus infections have been documented in many instances [24–26]. Enhanced accumulation of miR168 and altered expression of *Argonaute 1* (*AGO1*) mRNA was observed in CymRSV-infected plants [25]. Viral suppressors of RNA silencing (VSRs) from unrelated genera of RNA viruses were demonstrated to enhance expression of miR168 and this induction is suggested as an integral component of host defence suppression system [25,26]. In rice, Rice stripe virus (RSV) infection leads to expression of phased siRNAs and miRNAs derived from specific and conserved precursors respectively indicating the molecular interplay of siRNAs and miRNAs during infection process [27]. Deep sequencing studies in cotton infected by Cotton leafroll dwarf virus (CLRDV) not only showed deregulation of host miRNAs leading to symptoms and disease progression but altered expression of heterochromatin-associated siRNAs (ha-siRNAs) led to perturbation of epigenetic gene regulation [28]. Hence, these reports demonstrate the significance of host derived miRNAs in defining the outcome of phytopathogenic virus induced infection processes.

Soybean is an important oil seed crop, rich in nutritionally invaluable protein and biochemical components packed with nutraceutical benefits [29]. Worldwide cultivation and industrial applications of soybean is hampered due to infection and damage caused by 27 viruses [30]. In India, two major species of *Begomovirus* (*Mungbean yellow mosaic India virus* and *Mungbean yellow mosaic virus*) cause yellow mosaic disease (YMD) in soybean [31,32]. However, soybean is mainly grown as a major monsoon crop in the central Indian region where infection due to prevalent begomovirus Mungbean yellow mosaic India virus (MYMIV)- causes severe yield reduction (31–32). Developing MYMIV resistant soybean cultivar through molecular breeding or genetic modification approaches is a research priority to harness yield potential of soybean. Hence, identification and characterization of biomarkers linked to MYMIV resistance in soybean would greatly help in breeding MYMIV tolerant cultivar. Furthermore, it has been demonstrated that begomovirus infection causes alterations in expression levels of host miRNAs leading to developmental changes due to the miRNA mediated repression of host mRNAs [33]. Identification of endogenous plant miRNAs that are responsive to MYMIV infection and display binding capability to the viral genome-encoded transcripts could provide prospective small RNAs with potential use in virus resistance. Thus, this work aims to gain molecular insights regarding miRNA based responses towards MYMIV infection in two contrasting genotypes (JS335-susceptible to MYMIV and UPSM534-resistant to MYMIV) of soybean. Here we report MYMIV-sb infection causes significant changes in the expression levels of select conserved and putative antiviral miRNAs derived from soybean. Expression changes of corresponding target mRNAs also indicate that Argonaute homeostasis could possibly play critical role in conferring MYMIV resistance in soybean. Furthermore, studies involving host miRNA and target mRNA interactions point towards preferential targeting of begomovirus encoded transcript in conferring virus resistance trait of hosts.

2. Materials and methods

2.1. Plant growth and MYMIV infection

Seeds of soybean genotypes, UPSM 534 (resistant to MYMIV) and JS-335 (popular Indian cultivar, susceptible to MYMIV) were sown and germinated in plastic pots with a photoperiod setting of 16 h light/8hr dark under green house conditions. Seedlings of the soybean lines at the three leaf stage were inoculated using MYMIV-sb infectious clones (NRCS YIC- NRCS A2X and NRCS B2X) through in-house developed agro-inoculation procedure. Briefly, pricked stem nodes of young soybean seedlings at three leaf stage were inoculated with a mixture of overnight grown *A. tumefaciens* harbouring plasmids NRCS A2X and NRCS B2X (binary plasmids carrying head-tail tandem repeats of DNA A and DNA B genomic components of MYMIV-sb). Inoculated soybean leaves were collected and total DNA was extracted 10 days post inoculation (dpi) [34]. DNA from mock-inoculated leaves (*Agrobacterium* harbouring control binary plasmid which is devoid of viral DNA) of plants grown in controlled conditions was used as negative control. In order to assess successful infection due to MYMIV-sb, PCR assay and multiply primed rolling circle amplification (RCA) was carried out to detect virus infection and circular forms of viral DNA respectively following established protocol [32,35]. PCR amplification of conserved coat protein (CP) gene encoded by legume infecting begomoviruses was carried out using DNA template extracted from leaves of soybean genotypes and oligonucleotides RUGEMF1 5′ TGTGAGGGACCATGTAAAGTTC 3′ RUGEMR1 10: 5′ GCATGAGTACATGCCATATAC 3’ [31]. All the *Begomovirus* positive DNA samples were further analyzed through PCR assay described by Ramesh et al., 2016 [32]. PCR amplification of MYMIV specific coat protein (AV-1) gene was done using primer set MYMIV F 5′ GCATCAAGTCCGTGTACATTAC 3'and YMV R 5′CACAGGATTTGATGCATGAG 3’ [32]. Total genomic DNA extracted from inoculated, PCR positive and mock-inoculated plants were used in multiply primed rolling circle amplification (MPRCA) reaction. MPRCA mixture comprise approximately 50 ng of extracted total DNA, 2 μl of Phi 29 DNA polymerase buffer (10X), 2 μl of exo-resistant random primers (500 μM) and 2 μl of dNTPs (10 mM) (Fermentas, Massachusetts, USA) and the isothermal amplification reaction was conducted following protocol enumerated in Ramesh et al., 2017 [36]. Amplified products were digested with restriction enzymes *Xba* I and *Bam* HI (Fermentas) to release monomeric unit length of viral genomic components. PCR amplicons and restriction enzyme digestion products were run on 1% agarose gel followed by Et-Br staining and visualization.

2.2. Soybean miRNAs and target prediction

Conserved miRNAs enumerated by Bustos-Sanmamed et al., 2013 [37] were selected for expression profiling during MYMIV infection in soybean genotypes (Table 1). Identified and validated target mRNAs for the selected conserved miRNAs were obtained from miRBase database and cited references in Table 2 [38–42]. In addition, Mungbean yellow mosaic India virus-sb (MYMIV-sb) genome sequence (KC852204, KP828155) was used as a target query in the miRNA target prediction analysis using plant small RNA target analysis server (psRNATarget). MYMIV-sb genome and its encoded ORFs were submitted as input query (in FASTA format) in ‘pre-loaded small RNAs vs user-submitted transcripts’ interface of the server. All server parameters *viz*., length for complementarity scoring, maximum energy to unpair the target site (UPE) were set at default values whereas maximum expectation value (E) was set at 4.0 in order to improve the prediction coverage [43]. Mature *Glycine max* derived miRNAs, available in miRBase (release 21), were used

Table 1
Conserved *Glycine max* miRNAs and corresponding forward primers used in studying relative expression levels of miRNAs upon MYMIV infection. Expression levels of gma-miR156 was used as a reference miRNA for normalization.

miRNA	Primer Sequence 5′-3′
gma-miR156	CACACCAGATTGAGAGAGGC
gma-miR160	CATGCATACATATGTGTATG
gma-miR162	GTGAAGTCACTGGATGCAGC
gma-miR167	GAAGTTCGCAAAGGAAAAAG
gma-miR168	CACTGTGCGGTCTCTAATTC
gma-miR169	AAGAGGAAGAGAGAGTGATG
gma-miR393	GGAGGAGGCATCCAAAGGGA
gma-miR394	ATCATGAGGGTTTAGCAAAG
gma-miR396	TCATGGCTCTCTTTGTATTC
gma-miR398	CTCGGAGGAGTGAATCTGAG

for computational analysis to identify host derived miRNAs with potential capability to bind virus derived transcripts.

2.3. Extraction of small RNAs/Total RNA and reverse transcription

MYMIV-sb infected and mock-inoculated leaf samples were collected and flash frozen in liquid nitrogen for extraction of small and total RNA. In order to avoid any sampling or biological variations, leaves of three plants were pooled (mock-inoculated and infected leaves pooled separately) and ground in liquid nitrogen. About 150 mg of ground leaf tissue was used for extraction of miRNAs and total RNA using RNAeasy plant minikit (Qiagen). The extracted RNA was treated with RNAse free DNase I (Invitrogen, Carlsbad, CA, USA) for 30 min at 25 °C. The integrity of the RNA was checked by profiling it in 17% denaturing polyacrylamide gel electrophoresis, RNA concentration was measured using Nano drop prior to reverse transcription process. miScript reverse transcription kit (Qiagen) was used in cDNA synthesis of small RNAs following manufacturer's protocol. Similarly total mRNA was reverse transcribed using 2 μg of total RNA in a 50 μl reaction mixture using MMuLV reverse transcriptase (Thermo Fisher Scientific).

2.4. Quantitative real-time PCR

First strand cDNA obtained from reverse transcription process was used to assess the abundance of miRNAs and their putative target mRNAs. Relative quantitative expression of miRNAs and corresponding targets were performed in a RT-qPCR assay using dsDNA binding dye SYBR Green [44]. In the miRNA expression studies, miScript universal primer provided along with the miScript SYBR Green PCR Kit (Qiagen) was used as reverse primers whereas miRNA-specific oligonucleotides were employed as forward primers (Table 1). Similarly for studying the expression levels of target mRNAs, specific reverse and forward primers were designed and qRT-PCR performed using QuantiTect SYBR Green PCR Kit (Qiagen). Oligonucleotides employed in qRT-PCR studies are mentioned in Table 1 through Table 3. Expression status of gma-miR156 and *GmEF1A* (Eukaryotic elongation factor 1-alpha) was used as a reference genes for studying miRNA and target mRNA genes respectively [45,46]. qRT-PCR was carried out in Light Cycler R 480 II (Roche) and the amplification conditions were as follows: 95 °C for 2 min, 40 cycles of 95 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s qRT-PCR calculations were performed following delta CT value method [ΔΔCt = ΔCt of infected sample - ΔCt of control sample], following this fold change in the expression levels of miRNA: target pairs were obtained by $2^{-\Delta\Delta Ct}$. Dissociation curves were generated by performing thermal

Table 2
Target soybean mRNAs of selected conserved miRNAs and corresponding primer sets used for assessing relative expression patterns upon MYMIV infection. Expression of *GmEF1A* was used as a reference for qRT-PCR using the primers (GACCTTCTTCGTTTCTCGCA and CGAACCTCTCAATCACACGC).

miRNA	miRNA target transcript	Forward primer 5′-3′	Reverse primer 5′-3′	Reference
gma-miR160	Auxin responsive factor (ARF)	TmiR160F: AGCAGAGCCAAGAACAATGTC	TmiR160R: GTGGGCGATTACACTTCATTGG	Song et al., 2011 [38]
gma-miR162	Dicer homolog 1-like (DCL-1)	TmiR162F: GTCCAGCACATGCAAAGAGGTTT	TmiR162R: TCGCTCTCCAACACATTCATC	Zhai et al., 2011 [39]
gma-miR167	Zinc Finger family protein (ZF)	TmiR167F: CACCTGCCAAGTTTCAAAGAAGAG	TmiR167R: CCCTAGCTGTTCCAAGGGATTTC	Subramanian et al., 2008 [40]
gma-miR168	Argonaute-1(AGO)	TmiR168F: GCCATCCCACCGAATTTGAC	TmiR168R: GGACGGCTTGTACCCTGTA	Song et al., 2011 [38]
gma-miR169	Nuclear transcription factor Y subunit A-9-like (NUCLEAR FACTORY) (NF)	TmiR169F: GCATGTCAAGCGGCAAATCAT	TmiR169R: GATGGCCCAATCAATCATTCTTC	Subramanian et al., 2008 [40]
gma-miR393	AUXIN SIGNALING F-BOX protein (AUX S)	TmiR393F: TTGCGCACATGGCACTTC	TmiR393R: GGCTCCGACAGTCACTTCAC	Song et al., 2011 [38]
gma-miR394	NADP+	TmiR394F: GCTCGCCATTGTCGCTCAACT	TmiR394R: GGGTCTCGAACGCATGAAATAAAGTG	Song et al., 2011 [38]
gma-miR396	Growth-regulating factor 3-like (GRF)	TmiR396F: CCAGGCCCAAGAAATGAGAATG	TmiR396R: CCTCCGGCCCAATTCAAC	Kulcheski et al., 2011 [41]
gma-miR398	Copper superoxide dismutase	TmiR398F: CAATCGGTTGCCTGTCAACTG	TmiR398R: AGCTACTCTGCCACCAGCAT	Song et al., 2011 [38]; Dezulian et al., 2005 [42]

Table 3
Glycine max derived putative antiviral miRNAs, and their forward primers used for miRNA expression studies following MYMIV infection. The prospective antiviral miRNAs were identified based on small RNA target analysis using MYMIV genome and ORFs as a query sequence against soybean derived miRNAs. gma-miR156 was used as a reference gene for normalization of qRT-PCR based quantification.

Soybean miRNAs	viral genomic co-ordinates	Target genes or ORFs	Maximum energy required to unpair the target (UPE) kcal/mol)	Primer Sequences 5′-3′
gma-miR9743	KC852204 (1674–1693)	Complement to AC1	20.97	AAAATGCCATTCTCGTCTCTAAG
gma-miR2111	KP828155 (1385–1404)	Complement to BC1	16.557	GCTGGGTCCGGATTAGGTA
gma-miR4371	KP828155 (1718–1738)	Complement to BC1	22.769	AATGACGTGACAGACGGAAT
gma-miR4394	KP828155 (1287–1307)	Complement to BC1	16.016	GAGCTAGGTTTTGGGGTTGA
gma-miR5764	KP828155(317–336)	BV1	17.92	ACGGCCAAAGAAAGAAGGAA
gma-miR5785	KP828155(378–399)	BC1	16.469	ACGGACACCAACATGTGTCA
gma-miR9742	KP828155 (242–260)	BC1	22.484	CCTAATGCTA CAAAACAAAC

denaturing step to verify the specificity of amplification products.

2.5. Validation of target mRNA genes

Validation of soybean miRNA induced viral target gene cleavage was performed through 5′ Rapid amplification of cDNA Ends (5′ RACE) using SMARTer® RACE 5'/3 kit following the suggested protocol (Clonetech, CA, USA). Total RNA extracted after 8 h post inoculation was used in RACE experiments. The gene specific primers (GSPs) used for 5′RACE of MYMIV nuclear shuttle protein (NSP) gene are GSP1 BV1 5′ GGTTCCACAATTAGGCTATGC 3′ and GSP2 BV1 5′GGTCCTTGAACGTAGTCCACA3'. Similarly GSP for 5′ RACE of viral movement protein (MP) are GSP1 BC1 5′ AGGATCCAATGATGCCTCTG 3'and GSP2 BC1 5′ ATTGGGCCTGTCGTTACTTG 3'. RACE PCR products were cloned and sequenced following standard molecular biology protocols.

3. Results

3.1. MYMIV detection in soybean

Inoculated soybean plants displayed typical yellow mosaic symptoms associated with the disease in leaves (Fig. 1a). PCR amplification revealed Begomovirus infection in 12 out of 15 plants of JS-335 genotypes whereas DNA extracted from 4 plants among the 14 plants of UPSM-534 showed amplification product specific for legume Begomovirus. All the *Begomovirus* positive DNA samples were further analyzed through PCR assay specific for MYMIV coat protein (AV-1) gene using oligonucleotides MYMIV F 5′ GCATCAAGTCCGTGTACATTAC 3'and reverse primer (YMV R 5′CACAGGATTTGATGCATGAG 3′). In the PCR detection all 16 DNA samples (12 from JS-335 and 4 from UPSM-534) showed amplicon derived from coat protein gene specific to *Mungbean yellow mosaic India virus* (MYMIV) (Fig. 1b). Furthermore, multiply primed rolling circle amplification was performed using DNA template isolated from infected plants. MPRCA performs effective amplification of circular DNA genomes of begomovirus and the products of MPRCA were subjected to restriction enzyme digestion with *Xba* I and *Bam* HI. Restriction digestion of MPRCA products with enzymes *Xba* I and *Bam* HI indicated presence of ~2.7 kb, full length DNA-A and DNA-B genomic components of MYMIV-sb respectively (Fig. 1b). MYMIV specific PCR amplification and RCA derived products are presented for plant samples 3 & 11 (for JS335) and 1 & 4 (for UPSM534) (Fig. 1b). Thus, PCR and MPRCA based detection revealed that soybean plants are infected with MYMIV-sb due to agro-infection process.

3.2. Glycine max miRNAs and their targets

Conserved miRNAs derived from plants in general and soybean in particular displayed greater propensity for binding and repression of viral transcripts [47–50]. Based on these previous reports, soybean derived conserved miRNAs were selected for expression profiling during MYMIV infection (Table 1). Soybean derived mRNA targets for these selected miRNAs were obtained from psRNA target analysis and published references (Table 2). The target transcripts were transcriptional factors, enzymes involved in RNA silencing pathways, and other developmental processes in soybean. Notable among them are *Glycine max* endoribonuclease Dicer homolog 1-like and *Glycine max* protein argonaute 1-like that are target transcripts for soybean derived sRNAs, gma-miRNA162 and gma-miRNA168, that are involved in RNA silencing process.

Furthermore, *in silico* analysis identified some putative antiviral miRNAs derived from soybean against *Begomovirus* derived genomes and genes [48]. Building upon these findings, small RNA target prediction analysis using plant small RNA (psRNA) target server identified *Glycine max* derived miRNAs that showed binding ability to target *Mungbean yellow mosaic India virus* genomic regions (Table 3). Four soybean derived miRNAs *viz*., gma-miR9743, gma-miR2111, gma-miR4371 and gma-miR4394 were found to bind genomic region of MYMIV (Table 3) whereas, three miRNAs gma-miR5764, gma-miR5785 and gma-miR9742 showed sequence complementarity towards MYMIV genes such as BV1 and BC1 encoding nuclear shuttle protein (NSP) and movement protein (MP) respectively.

3.3. Expression changes of conserved microRNAs during MYMIV infection

Among the selected nine conserved soybean miRNAs, three miRNAs (gma-miR168, gma-miR394 and gma-miR396) were found to be upregulated in the cultivar JS 335 during MYMIV infection (Fig. 2). The upregulation varied from 1.45 fold (gma-miR396) to 5.89 fold (gma-miR168). All other conserved miRNAs such as miR160, miR162, miR167, miR169, miR393, miR398 were downregulated to varying degrees upon MYMIV infection (Fig. 2). In another soybean genotype (UPSM-534), MYMIV inoculation caused upregulation of three miRNAs including a miRNA (gma-miR394) that was found to be upregulated in JS335 during viral infection. MYMIV infection in UPSM 534 caused upregulation of miR169 (5.7 fold) followed by miR394 (4.76 fold), and miR398 (4 fold). Hence differential expression of conserved miRNAs between both the genotypes was observed with gma-miRNAs, miR168, miR169, miR396 and miR398. Hence, the miR396 shown to be over expressed in JS335 was found to be downregulated along with

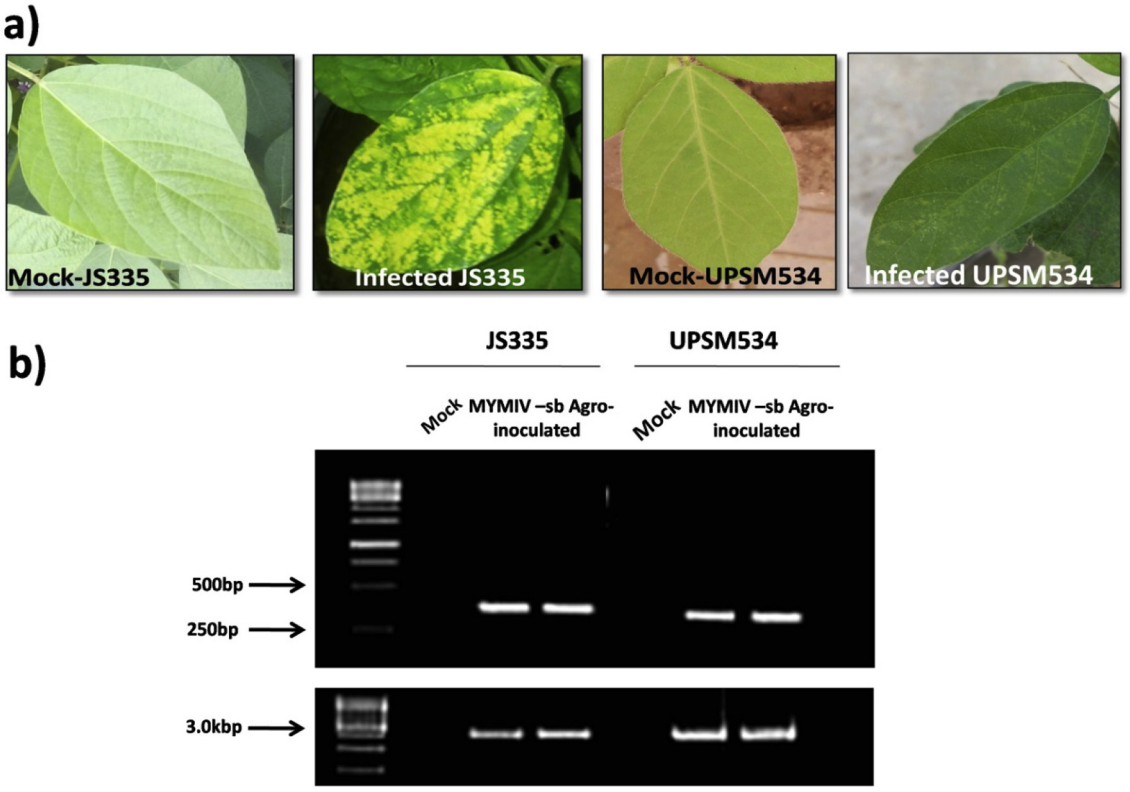

Fig. 1. a) Agro-inoculation of MYMIV-sb and mock-inoculated leaf samples of soybean genotypes JS335 (susceptible to YMD) and UPSM534 (resistant to YMD). Agro-inoculation of soybean seedlings was done at three leaf stage using MYMIV-sb infectious plasmids whereas Agrobacterium harbouring empty plasmid was used as mock. b) Detection of viral DNA in susceptible and resistant soybean genotypes following agro-inoculation. PCR detection of MYMIV coat protein gene (CP) using DNA extracted from agro-inoculated plant leaf tissue and MYMIV-sb specific primers followed by rolling circle amplification and restriction digestion (*Xba* I) to resolve MYMIV DNA-A specific ~2.7 kb viral genomic DNA is depicted.

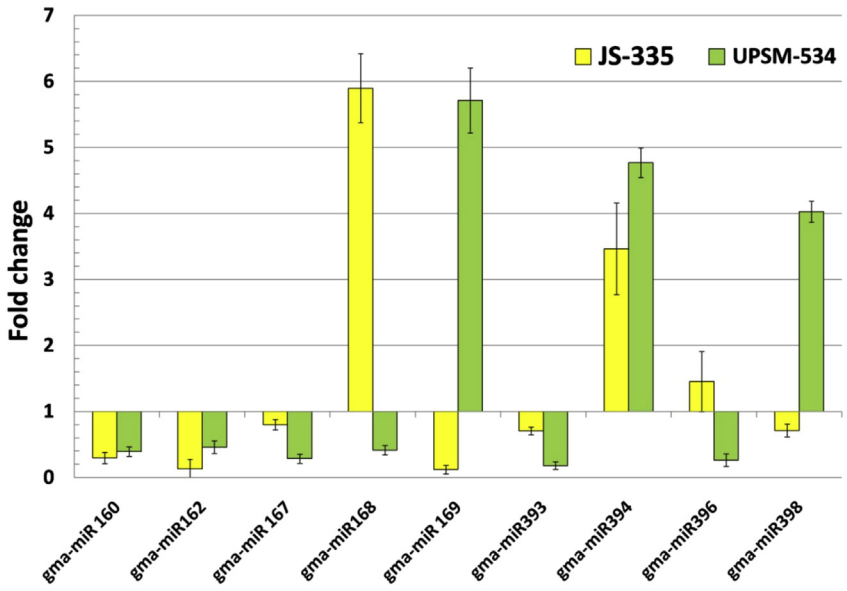

Fig. 2. Fold change in expression of conserved miRNAs in soybean genotypes (JS335 and UPSM534) following MYMIV-sb agro-infection. Expression profiling of selected soybean miRNAs by qRT-PCR was performed 10 days following agro-infection with MYMIV-sb. Mean fold change in expression of miRNAs from three biological replicates is depicted where error-bars reflect calculated SD. Normalization of qRT-PCR data was done with reference to gma-miR156.

other miRNAs like gma-miR160; gma-miR167 and gma-miR393 in the genotype UPSM 534.

3.4. Expression changes of corresponding miRNA target transcripts

In general, the target mRNAs and their respective conserved

miRNAs showed inverse relationship with regard to fold changes in expression levels *i.e.*) miRNAs that were upregulated showed repression of target mRNA expression, whereas converse was observed with miRNAs that were downregulated. Among the miRNA target genes profiled in the cultivar JS335, three mRNA targets *viz*., NADP+ (4.6), AGO (4.9) and GRF (9.7) were downregulated, whereas rest of the mRNA targets [ARF (5 fold), DCL (4.93 fold), ZF (6.63 fold), NF (6.8), AUX S (7.4) and CuSOD (5.58)] were found to be upregulated (Fig. 3). The expression pattern of target genes in JS335 suggests that transcripts involved in RNA silencing process (AGO), and other genes involved in developmental aspects are downregulated (GRF and NADP+). However mRNAs involved hormonal response and signaling pathways (ARF, AUX S) or transcriptional factors (ZF and NF), genes involved in oxidative stress response (CuSOD) are upregulated during MYMIV infection in JS335. Notably, among the genes involved in RNA silencing though DCL was upregulated, mRNA encoding the slicer, AGO, was downregulated in JS335. UPSM 534 derived mRNA targets also followed similar regulation status under MYMIV infection however, contrasting expression status of some of the target genes was also observed (Fig. 3). miRNA target genes such as AGO (7.65) and GRF (4.37 fold) were upregulated and NF (2.2 fold) and CuSOD (2.6 fold) were downregulated whereas contradictory expression status was observed for those target genes in JS335. Remarkably, among the genes involved in RNA silencing pathway, DCL was upregulated in both the genotypes however AGO was found to be upregulated in UPSM 534 in contrast to down regulation observed in JS335. Genes involved in hormonal signaling (ARF and AUX S) and zinc finger TFs were over expressed in both the genotypes, suggesting the importance of hormone mediated defence response besides RNA silencing.

3.5. Expression profiling of putative antiviral miRNAs

Seven soybean derived miRNAs have been selected (gma-miR2111, gma-miR4394, gma-miR4371, gma-miR9743, gma-miR5764, gma-miR5765 and gma-miR9742) for studying their expression pattern under MYMIV infection in both the soybean genotypes. These putatively antiviral miRNAs have been identified based on their ability to bind MYMIV genome and/or genes due to sequence complementarity and *in silico* sRNA target prediction analysis. Expression studies revealed that all the seven soybean miRNAs were upregulated during MYMIV infection in both JS-335 and UPSM-534 (Fig. 4). None of the miRNAs under scrutiny showed less than 2 fold change in upregulation. In general, putatively antiviral miRNAs derived from UPSM534 showed relatively high expression/upregulation compared to miRNAs derived from MYMIV susceptible genotype JS335 with the exceptions being gma-miR4394 and gma-miR9742. Thus, the latter two miRNAs in the cultivar JS335 showed more upregulation ie) 6.19 and 7.2 fold change in over-expression compared to corresponding miRNAs derived from UPSM 534. Notable among the UPSM-534 derived miRNAs were gma-miR2111 and gma-miR5764 that respectively displayed 8.7 and 8.9 fold change in over-expression under MYMIV infection (Fig. 4).

3.6. Soybean miRNAs target MYMIV transcripts

Soybean derived miRNAs (gma-miR5764, gma-miR5785 and gma-miR9742) showed sequence complementarity towards MYMIV ORFs such as BV1 and BC1 encoding nuclear shuttle protein (NSP) and movement protein (MP) respectively (Fig. 5a). Further, these miRNAs also showed over expression in both the soybean genotypes 8 h after MYMIV inoculation. In order to further assess antiviral activity of these miRNAs, the point of cleavage of viral mRNAs (BV1 and BC1) was assessed through 5′ RACE studies. Cleavage products of two viral mRNAs (BC1 and BV1) were studied using total mRNA extracted from infected soybean lines UPSM 534 and JS 335. BC1 derived mRNA species of 312 bp in length, resulting from the cleavage activity of gma-miR5785, was found to

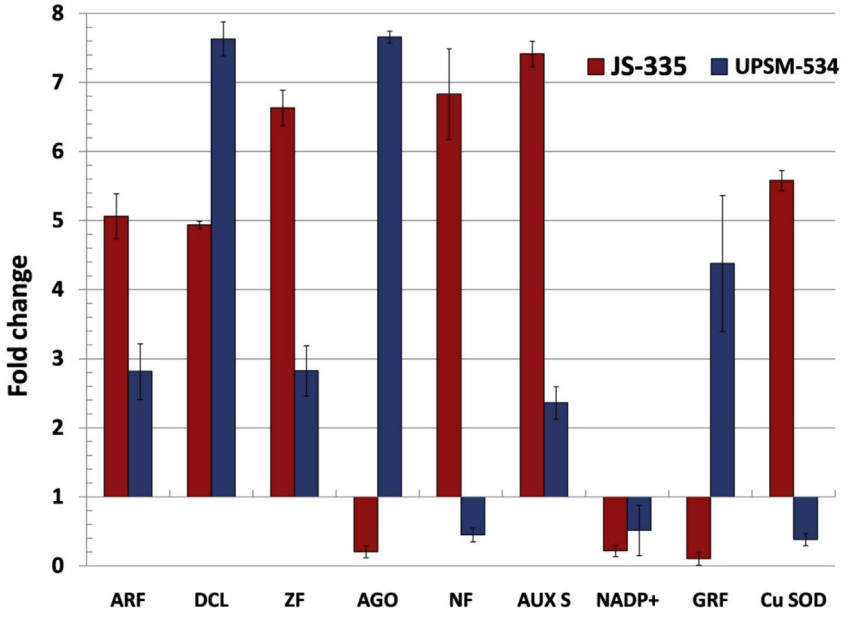

Fig. 3. Fold change in expression levels of target mRNAs of conserved miRNAs in soybean genotypes (JS335 and UPSM534) during MYMIV-sb infection. Expression profiling of target mRNAs by qRT-PCR was performed at the same time point as their corresponding miRNAs (10 days post agro-infection with MYMIV-sb). Mean fold change in expression of target genes from three biological replicates is depicted where error-bars reflect calculated SD. Normalization of qRT-PCR data was done with reference to the expression of *GmEF1A* (ARF-Auxin response factor; DCL-Dicer-like; ZF- Zinc Finger TF; AGO-Argonaute; NF- NUCLEAR FACTORY; AUX S- AUXIN SIGNALING F-BOX protein; NADP + - NADP+; GRF- Growth-regulating factor; CuSOD- Copper super oxide dismutase).

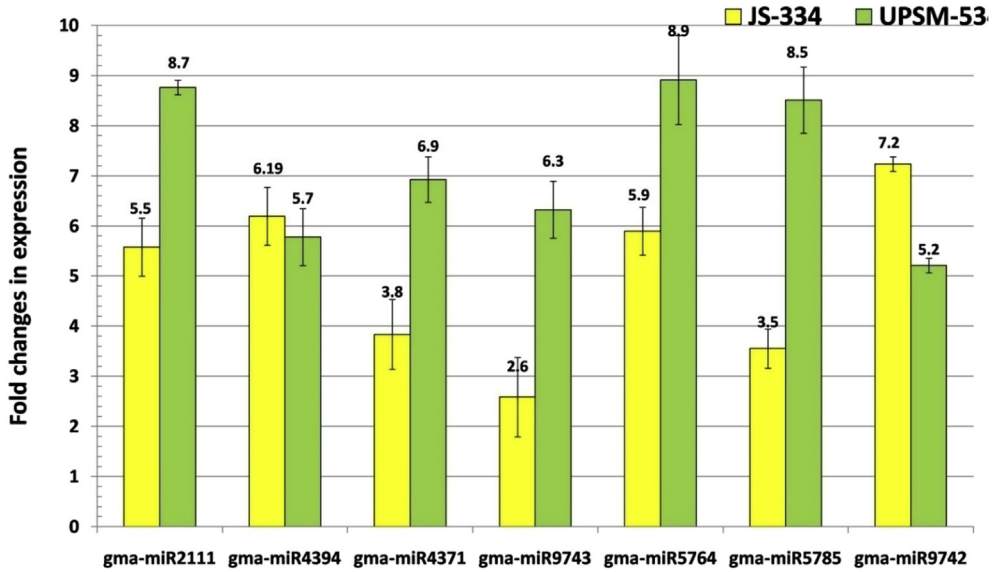

Fig. 4. Relative expression analysis of putative antiviral miRNAs derived from soybean genotypes JS335 and UPSM 534 during MYMIV infection. Expression profiling of selected soybean miRNAs by qRT-PCR was performed 10 days following agro-infection with MYMIV-sb. Mean fold change in expression of miRNAs from three biological replicates is depicted where error-bars reflect calculated SD. Normalization of qRT-PCR data was done with reference to expression level of gma-miR156.

accumulate in UPSM 534 and JS335 plants. Cloning and sequencing of RACE PCR product confirmed expected size of fragment of viral origin due to soybean miRNA mediated cleavage activity. Expectedly, the amplicon was absent in the mock-inoculated plants. However, comparison of levels of accumulation of BC1 derived transcript was found high in infected UPSM 534 plants compared to JS335 plants. Moreover, seven out of eight clones sequenced showed viral mRNA amplicons specific to expected point of miRNA mediated cleavage in UPSM 534 whereas, in the susceptible cultivar JS335, one out of nine clones sequenced showed desired size of viral mRNA amplicon (Fig. 5b). It thus proved the direct antiviral activity of gma-miR5785 against the viral encoded movement protein mRNA in soybean during MYMIV infection. However, cleaved PCR product of virus derived BV1 transcript was not detected in RACE PCR studies conducted in both soybean genotypes. Thus, validation of targets for host miRNA mediated cleavage of viral mRNA revealed that UPSM 534 derived miRNA gma-miR5785 effectively cleaves viral movement protein mRNA compared to corresponding miRNA in JS335- a susceptible line.

4. Discussion

In an era of small non-coding RNAs (ncRNAs), understanding the role of plant derived small RNAs in conferring defence against virus invasion is a main research priority. Among various small ncRNA species, small interfering RNAs (siRNAs) and artificial miRNAs (amiRNAs) have been widely deployed to confer virus resistance in crop plants through genetic modification approaches. siRNA based antiviral defence mechanism in plants have been well documented whereas the role of plant derived miRNAs in viral defence and/or susceptibility warrants a deeper study. Thus, studies exploring expression dynamics of plant miRNAs in response to pathogenic virus infection would provide insights into host's defence response, molecular events in disease progression and possibly sncRNAs with antiviral potential. Accordingly, reports have revealed that expression levels of host miRNAs are widely modulated during begomovirus infection in plants leading to changes in host gene expression [33,50]. On the other hand, host miRNAs have been demonstrated to display propensity to bind and repress begomovirus genomes and encoded ORFs [48,51]. Similarly, beta satellites associated with begomoviruses also incite diverse gene regulatory changes by altering expression pattern of miRNAs in *Nicotiana benthamiana* during virus infection [52]. These reports, hence, suggest miRNAs play defining roles in altering the gene regulatory mechanism of host plants and also presumably in conferring resistance against begomovirus infection.

Mungbean yellow mosaic India virus (MYMIV) (Begomovirus) is a serious pathogen of soybean cultivated in central India [31,32]. In the absence of MYMIV resistant soybean cultivar suitable for central Indian region, investigating soybean miRNAs responsive to MYMIV infection not only help gain understanding of virus-host molecular interactions but also would identify sRNA functional biomarkers associated with MYMIV resistance in soybean. Genetic mapping and validation of these resistance conferring loci in soybean mapping population could greatly improve molecular breeding approaches to impart MYMIV resistance. Furthermore, it has been proposed that host derived conserved miRNAs are evolutionarily selected and maintained in plant kingdom to confer defence against invading pathogens like viruses [47,48]. In addition, soybean miRNAs have been shown to display proclivity to repress MYMIV genome and genes and are called as putative antiviral miRNAs (49). In this investigation, we studied the expression pattern of host derived conserved miRNAs and putative antiviral miRNAs in soybean during MYMIV-sb infection. In order to gain genotypic response to MYMIV infection, two soybean lines [JS335 (S) and UPSM534 (R)] that were showing contrasting disease resistance trait were studied.

Our study indicates that conserved miRNAs are differently expressed in both the soybean genotypes upon MYMIV infection implying inherent diversity in the molecular response of contrasting soybean genotypes to viral infection. In the resistant soybean genotype (UPSM 534) miRNAs 398 and miRNA 169 were upregulated and miRNA168 was found to be downregulated whereas strikingly different miRNA expression pattern was observed in susceptible cultivar JS335. It thus suggests that corresponding miRNA target transcripts, copper super oxide dismutase (CuSOD), NUCLEAR FACTORY (NF) and Argonaute1 (AGO) respectively (Fig. 3) could presumably play a considerable role in virus

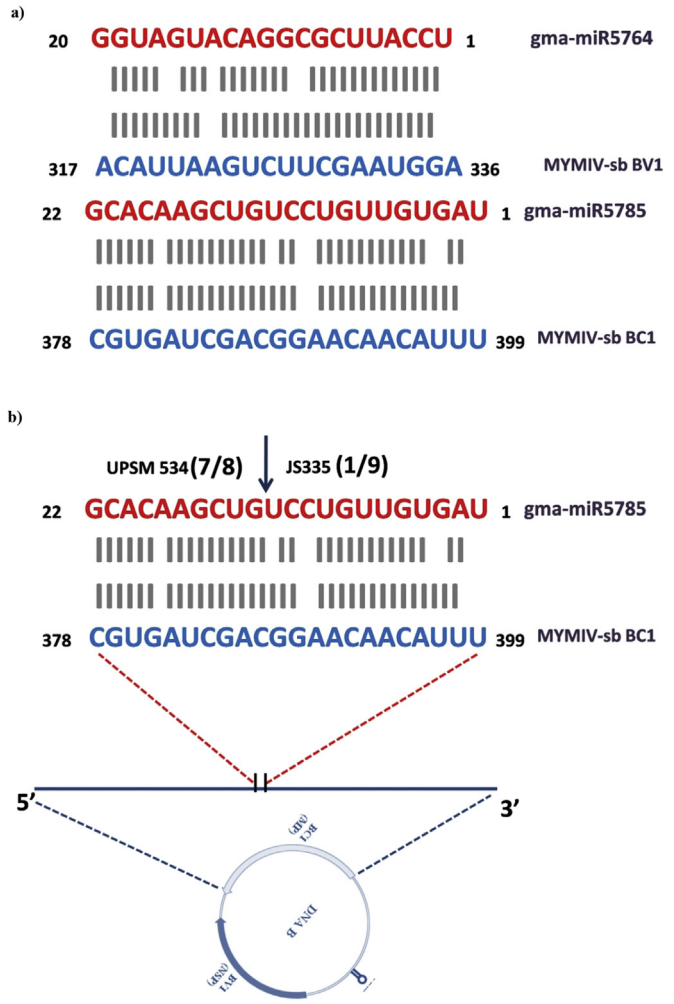

Fig. 5. Soybean derived miRNAs and MYMIV encoded movement genes. a) Plant small RNA target analysis identified and predicted potent soybean derived miRNAs (gma-miR5785 and gma-miR5764) to target MYMIV derived genes BC1 and BV1 encoding nuclear shuttle protein and movement protein respectively. b) RACE-PCR studies identified BC1 specific cleaved product due to the targeting activity of gma-miR5785 in UPSM534 (7 clones out of 8) and JS335 (1 of 9) (The co-ordinates of soybean miRNA and virus derived BC1 mRNA are presented).

resistance. Among these, argonaute (AGO1) proteins encoded by target mRNA has been revealed to be involved in RNA silencing process [53]. In the susceptible soybean line JS335, genes involved in hormonal signaling pathways, oxidative stress and TFs such as Auxin response factor (ARF), AUX S- AUXIN SIGNALING F-BOX protein, CuSOD- Copper super oxide dismutase, Zinc Finger (ZF), and Nuclear Factory (NF) are upregulated during MYMIV infection. UPSM 534 also showed upregulation of hormone signaling related genes ARF and AUX S, but down regulation of CuSOD was observed indicating inherent differences. Notably, both the genotypes showed expression of DCL transcripts however AGO-1 was over-expressed in UPSM534-which is resistant to MYMIV infection-as against its downregulation in JS335 (susceptible cultivar) indicating the significance of AGO homeostasis in antiviral resistance. Previous report has demonstrated that soybean genotypes activate genes involved in methylation and small RNA processing including Argonaut 4 and Dicer-1 homologue during early phase of MYMIV infection [54]. Hence, it is speculated that over expression of Argonaute protein (due to the concomitant decrease in expression of miRNA 168) in the resistant line UPSM534 might play a definitive role in conferring MYMIV resistance. Similarly, the function of AGO homeostasis in providing virus resistance has been proven during Soybean mosaic virus (SMV) infection in soybean [55]. SMV have RNA as their genomic component hence, slicing of viral genome derived dsRNA due to the activity of AGO1 protein could play a greater role in virus resistance whereas, MYMIV comprise ssDNA genomic components. Nevertheless the characteristic features such as overlapping ORFs and bidirectional promoters of genomes of Geminivirus along with read through transcription are the factors that could potentially trigger the process of RNA silencing [56,57]. Thus our results once again corroborate the observation that massive activation of innate immunity and specific defence related pathways in soybean plants during early phase of MYMIV infection [54]. A recent NGS based study revealed differential gene expression of ARF, SOD genes, among others, upon MYMIV infection in another legume crop *Vigna mungo* implying that the findings presented here are in consistence with global transriptomic changes [44].

In tomato: Tomato leaf curl New Delhi begomovirus virus interaction number of miRNAs and miRNAs* have been identified with propensity to bind virus genomes and its genes [50]. Further, expression analysis of tomato derived miRNAs during begomovirus Tomato leaf curl New Delhi virus (ToLCNDV) infection showed tissue specific accumulation of miR159/319 and miR172 suggesting important role for these miRNAs in tomato leaf curl disease progression [51]. On the otherhand, in legume *Vigna unguiculata*, *Tospovirus* infection was shown to instigate host miRNA mediated programmed cell death [58]. In addition, novel phased miRNA expression was also observed in rice during infection with Rice stripe virus (RSV) [27]. Multiple virus infections (Plum pox virus, Potato virus X and Potato virus Y) profoundly alter host miRNA expression in *N. benthamiana* [59]. These previous reports propose that there could be significant changes in the expression levels of soybean miRNAs upon MYMIV infection. Expectedly, expression profiling of some of the soybean derived antiviral miRNAs during MYMIV infection showed upregulation indicating the significance of miRNAs in defining the disease reaction. These putatively antiviral miRNAs were identified employing small RNA target analysis based on their proclivity to bind MYMIV genome and mRNAs (Table 3). Furthermore, viral derived transcripts were assessed for host miRNA directed cleavage action and accumulation of cleaved target transcripts using RACE PCR technique. This RACE-PCR study has revealed gma-miR5785 mediated targeting and cleavage of MYMIV derived transcript BC1 encoding viral movement protein. Plant derived miRNAs have been demonstrated to show antiviral activity by repressing plum pox virus replication [60]. Similarly, 6 different novel miRNAs have been shown to be upregulated and play a role in plant's defence mechanism during grapevine infecting viruses [61]. Besides correlating miRNA accumulation to severity of symptoms and disease progression [62], miRNA mediated complex reprogramming of gene regulatory mechanism has also been predicted to occur during TMV infection in *N. tabaccum* [63]. The complex interplay of begomovirus and host *Jatropha* derived miRNAs ultimately decides the outcome of pathogen: plant interactions [64]. Furthermore conserved miRNA families derived from plants are implicated with increased antiviral capabilities [47,49,50].

Considering the expression pattern of conserved miRNAs and their target mRNAs (Fig. 6) in MYMIV agro-infected soybean it was observed that genes involved in hormonal signaling pathways (ARF, AUX S) RNA silencing based defence mechanism (DCL), and TFs (ZFs) were upregulated. Contrarily downregulation of transcript encoding NADP+ was observed in soybean. The difference between susceptible and resistant reaction of soybean genotype could thus presumably be explained based on the differential behavior of genes involved in RNA silencing activity, oxidative stress (CuSOD)

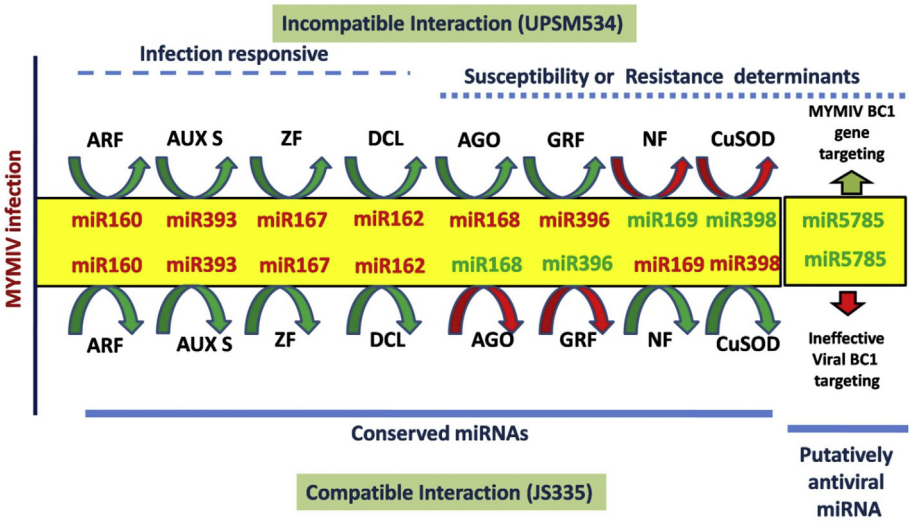

Fig. 6. Model proposed for miRNA mediated responsiveness and gene regulatory changes during MYMIV infection in soybean. (Red coloured arrows and letters indicate down-regulation, green indicates upregulation). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and TFs such as (Nuclear Factory, GRF). Susceptible reaction characteristically has miRNA mediated repression of AGO-1and GRF transcripts concomitant with miRNA mediated over expression of NF and CuSOD genes. Contrastingly, UPSM534 showed over expression of AGO-1 and GRF eventhough NF and CuSOD genes are downregulated. Hence, a model has been proposed to delineate molecular response of soybean towards MYMIV infection (Fig. 6). Infection responsive soybean miRNAs are basal sncRNAs that displayed altered expression during MYMIV infection however disease reaction (ie susceptibility or resistance of the genotype) is likely to be determined by expression levels of miRNAs *viz*., gma-miR-169, gma-miR-168, gma-miR-396, gma-miR-398 and gma-miR-5785 (Fig. 6).

5. Conclusion

In order to gain molecular insights, expressional changes of soybean derived miRNAs and their respective targets were studied in contrasting soybean genotypes [JS335 (S) and UPSM 534 (R)] during begomovirus (MYMIV) infection. This study demonstrated that expression levels of conserved and putatively antiviral miRNAs of host varied due to inherent genotypic differences of soybean lines. The upregulation of AGO-1 and concomitant host miRNA (gma-miR5785) mediated targeting of viral genome derived BC1 transcript during early stage of MYMIV infection in the resistant line UPSM534 could perhaps elucidate the underlying molecular basis of MYMIV resistance in soybean. Thus, a combination of miRNA mediated AGO homeostasis and targeting of viral movement protein might be involved in determining the resistance reaction in soybean against MYMIV infection.

Author contributions

SVR Conceived and designed the study, BSC, GK performed the experiments, SP & SC analyzed and interpreted data, SVR, SP & BSC wrote the manuscript, All authors read and approved the manuscript.

Conflict of interest

Authors declare that they have no conflict of interest.

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