Soybean (*Glycine max*) microRNAs display proclivity to repress *Begomovirus* genomes

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Small non-coding RNAs are important effector molecules in response to pathogen invasion in plants and animals. We conducted *in silico* analysis of the DNA genomes of two distinct species of genus *Begomovirus* (family Geminiviridae) – *Mungbean yellow mosaic India virus* (MYMIV) and *Mungbean yellow mosaic virus* (MYMV) – that infect soybean using a microRNA (miRNA) target prediction algorithm, plant small RNA target analyzing server. MYMV displays greater vulnerability to plant miRNAs with 99 miRNAs targeting its genome, whereas 70 miRNAs appear to be targeting the MYMIV genome. miRNAs derived from *Glycine max*, *Glycine soja* and *Cajanus cajan* display 63, 18, and 8 potential target sites on the begomovirus genomes. Among the non-host plants begomoviruses exhibit seven and six potential target sites for *O. sativa*, and *P. trichocarpa*-derived miRNAs respectively. Begomovirus ORFs encoding viral movement proteins reveal greater vulnerability for *G. max*-derived miRNA binding and repression. Computational analysis with ssDNA animal virus genome as negative control sequences further emphasizes that plant miRNAs preferentially target begomovirus genomes. Nine prospective soybean-derived miRNAs targeting begomovirus genes have been shown to play a role in host–microbe interactions and abiotic stress responsiveness. The study thus provides *in silico* evidence for the plant-derived miRNAs in antiviral immunity.

Keywords: Antiviral resistance, *Begomovirus*, microRNA, soybean.

SMALL non-coding RNAs (ncRNAs) comprising small interfering RNAs (siRNAs) and microRNAs (miRNAs) are gaining importance in the context of RNA-mediated gene regulation in plants and animals. miRNAs form a major component of the ncRNA repertoire in plants and regulate host gene expression pathways[1], including adaptation to viral infections[2–4]. Soybean is infected by more than 27 viruses that hinder its cultivation and industrial applications[5]. In India, two begomoviruses, *Mungbean yellow mosaic India virus* (MYMIV) and *Mungbean yellow mosaic virus* (MYMV), cause yield loss in legumes to the tune of 300 million USD[6]. Begomovirus infection generates perturbations in host miRNA expression levels, and represses plant developmental genes[3]. Identification of endogenous plant miRNAs that display binding capability to the viral genome-encoded transcripts could provide prospective small RNAs with potential use in virus resistance.

Genomes of MYMIV (EU523045, EU523046) and MYMV (AJ421642, AJ582267) that infect soybean were obtained from GenBank. Mature miRNAs of soybean and other plants from miRBase (Release 21, June 2014)[7] and hosted in plant small RNA target analysis server[8] were used for computational analysis. In addition, miRNAs discovered from other begomovirus hosts such as *Cajanus cajan*, *Glycine clandestine*, *Glycine soja*, *Phaseolous aconitifolius*, *Phaseolous vulgaris*, *Vigna mungo*, and *Vigna unguiculata*, were also used[9–11]. Genome sequence of equivalent animal-infecting ssDNA virus was used as negative control (Parvovirus: GU938300). Computational predictions of miRNA cross silencing activity against viral transcripts were accomplished using plant small RNA target analysis server (psRNATarget)[8]. Statistical analysis on Wilcoxon signed rank *t*-test was performed for comparing the significance of plant miRNA-based begomovirus genome repression with the effect of plant small RNAs on the animal virus genome (Parvovirus: GU938300).

The plant small RNA target server predicts miRNA target sites on the query sequence in both sense and reverse complementary orientations. This attribute of the server is suitable for analysis of the begomovirus genome since viral genome encodes for functional transcripts on both viral and complementary sense strands. miRNAs derived from 16 and 18 plant species have exhibited potential cross-silencing activity against the genomes of MYMIV and MYMV respectively (Figure 1 *a* and *b*). Among the miRNAs derived from plant species that are natural hosts of begomoviruses, 49 miRNAs have displayed propensity to target the MYMIV genome, whereas 65 miRNAs have displayed proclivity to repress the MYMV genome. Among miRNAs derived from non-host plants, the MYMIV genome is potentially targeted by 21 miRNAs whereas 34 miRNAs target the MYMV genome (Figure 1 *a*). Thus, overall, 70 miRNAs exhibited propensity to target the MYMIV genome and 99 miRNAs showed targets in the MYMV genome (Figure 1 *b*). However, analysis of miRNA target sites revealed 61 and 72 sites on MYMIV and MYMV genomes respectively. The difference in the number of putatively positive miRNAs and hits on the viral genome is because many miRNAs repeatedly target the same viral genome sequence (Table 1). Among the miRNAs derived from natural hosts, 68, 23 and 14 sRNAs derived from *Glycine max*, *G. soja* and *C. cajan* respectively, revealed antiviral role against the begomoviruses. However, sRNAs from the respective plants displayed 63, 18 and 8 potential target sites respectively, on the begomovirus genomes. miRNAs known in other host species like *P. aconitifolius*, *P. vulgaris*,

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V. mungo and *V. unguiculata* revealed nine potential antiviral miRNAs. Among the non-host plants, *Populus trichocarpa* miRNAs exhibited the highest number of hits on the begomovirus genome followed by *Oryza sativa* with 20 and 8 hits respectively. Begomovirus genomes exhibited 7 and 6 potential target sites for *O. sativa* and *P. trichocarpa*-derived miRNAs, respectively (Table 1). Comparing the genomic components, DNA B of MYMIV and MYMV attracted 43 and 62 hits whereas DNA A genome revealed 27 and 37 hits respectively. Among the begomovirus hosts, percentage of positive miRNAs was found to be highest with *P. aconitifolius* (18.18%) and *G. soja* (15.86%) followed by *G. max* (10.9%), *V. mungo* (6.06%) and *C. cajan* (5.55%). In the non-host plants, miRNAs from *Cyanara cardunculus* (5.26%) showed highest percentage of positive miRNAs followed by *Populus* (4.987%) and *Brassica rapa* (4.65%). Analysis of matching percentage – which is the measure of positive miRNAs with reference to the number of potential candidate miRNAs and virus genome – also revealed a similar trend. miRNAs from *P. aconitifolius* (1.6%) and *G. soja* (1.46%) among the natural hosts, and *Cyanara* (0.48%) and *Populus* (0.46%), among the natural non-hosts, displayed higher matching percentage (0.486) (Table 1).

Various ORFs-encoded by MYMIV and MYMV were analysed for their vulnerability to plant miRNAs. The ORFs of MYMV showed more miRNA hits (39) and target sites (24) than the MYMIV ORFs which showed 23 hits and 18 target sites (Figure 2). Among the MYMIV-coded ORFs, AV1 displayed more vulnerability (eight hits) followed by BC1 (four hits). Soybean-derived miRNAs were found to profusely target the MYMIV ORFs. Soybean miRNAs, gmamiR5785, gma-miR5764 and gma-miR5734 were identified to target viral movement protein (MP) and nuclear shuttle protein (NSP) respectively. MP and NSP are involved in intercellular and intracellular movement of viral DNA; hence both the genes are suitable targets for sncRNA-based gene silencing of the begomovirus. However, MYMIV-encoded AC1 ORF, embedded ORF AC4 encoding a viral suppressor protein (VSP), and an overlapping ORF AC 2 encoding transcriptional activator protein (TrAP) were potentially targeted by five miRNAs, but none from soybean (osa-miR5833, ptc-miR6433-5p and aly-miR3444a-5p). miRNA targeting the viral genome (DNA B) suggests that ORF BC1-coded MP is also targeted (three miRNA hits: pco-mir235, ahy-miR3513-3p, mtr-miR5227) by other plant miRNAs (Figure 2).

Among the MYMV-encoded ORFs, AC2 displayed more vulnerability (nine hits: miR 172 family from cca, vmu, vun; ath-miR822, aly-miR172e-3p, nta-miR6150)

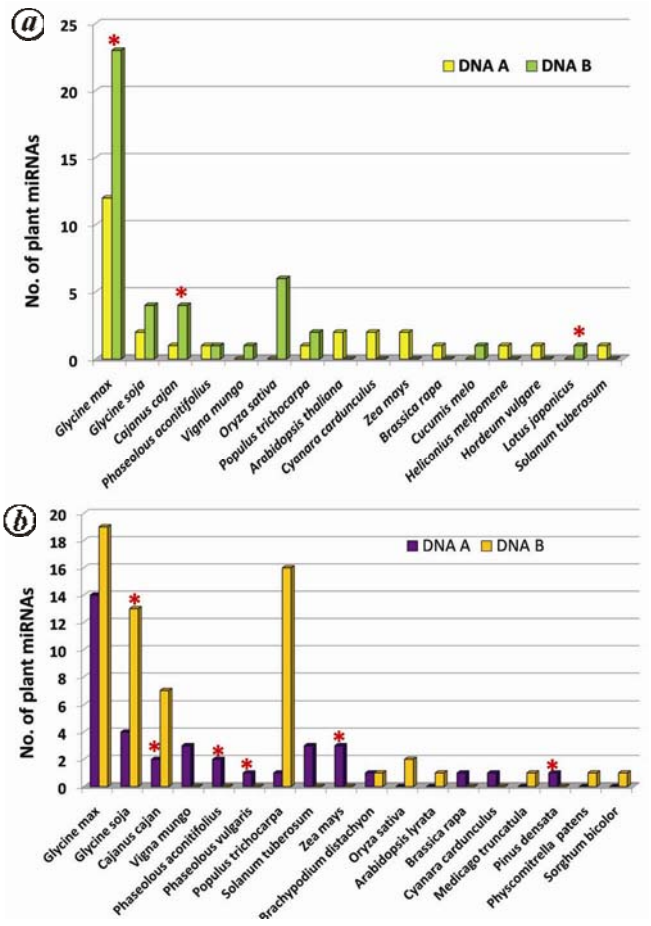

Figure 1. Number of plant microRNA (miRNA) hits on the DNA A and DNA B genomes of (***a***) *Mungbean yellow mosaic India virus* (MYMIV) and (***b***) *Mungbean yellow mosaic virus* (MYMV). *Plants showing conserved miRNA families: (***a***) (ca-miR167; Cca-miR2111, gma-miR2111; gma-miR2111; gma-miR4394 and lja-miR2111)] and (***b***) (Cca-miR172; Cca-miR394, gso-PN-miR172, Pac-MIR159, Pvu-MIR159, vun-MIR172, pde-miR159, vmu-miR159; vmu-miR172 and zma-miR394; zma-miR167).

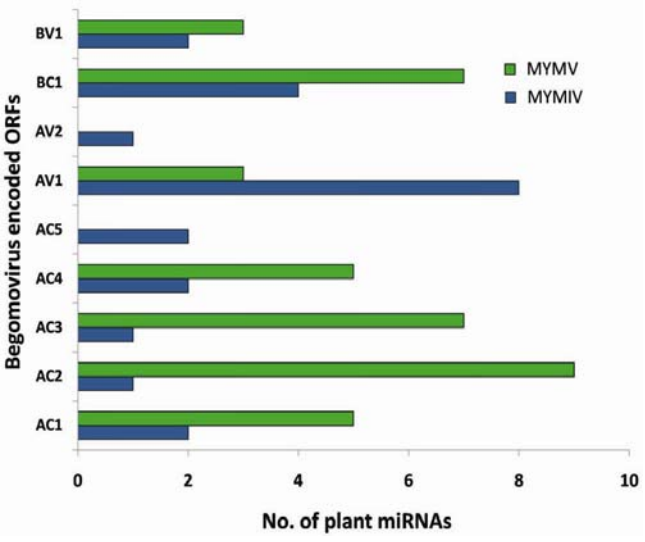

Figure 2. Number of plant miRNA hits on MYMIV and MYMV encoded genes.

Table 1. Summary of plant miRNA target analysis on begomovirus genomes

	Natural hosts						Non-hosts															
Plant species	cca	gma	gso	pac	pvu	vmu	aly	ath	bdi	bra	cyca	cme	hme	hvu	lja	mtr	osa	pde	ppt	ptc	sbi	stu	zma
Number of miRNAs	144	628	145	22	56	66	384	337	464	43	57	120	97	69	67	756	713	30	280	401	241	343	321
Number of miRNA hits on viral genome	14	68	23	4	1	4	1	2	2	2	3	1	1	1	1	1	8	1	1	20	1	4	5
Number of miRNA target sites	8	63	18	4	1	4	1	1	2	2	3	1	1	1	1	1	7	1	1	6	1	2	3
Positive miRNAs (%)	5.555	10.914	15.86	18.18	1.785	6.0606	0.26	0.593	0.431	4.651	5.263	0.833	1.03	1.449	1.492	0.132	1.122	3.333	0.357	4.987	0.414	1.166	1.557
Matching percentage	0.899	1	1.465	1.600	0.165	0.5601	0.024	0.0548	0.0398	0.4298	0.4864	0.07701	0.0952	0.1339	0.1379	0.012225	0.103	0.308	0.033	0.4609	0.03834	0.10778	0.1439

Positive miRNAs (%): Percentage of positive miRNAs among the miRNA repertoire of the respective plant species.
Matching percentage: [No. of candidates/size of virus genome × no. of miRNAs] × 100.

Table 2. Statistical analysis of algorithm parameters to compare plant miRNAs versus begomovirus against plant miRNAs versus animal-infecting ssDNA virus

Parameters		Plant miRNAs versus begomoviruses		Plant miRNAs versus ssDNA animal virus (negative control)	Pairwise Wilcoxon *t*-test values	
		MYMV (I)	MYMIV (II)	Parvovirus (III)	I versus III	II versus III
Free-folding energy (kcal/mol)	Minimum	11.435	5.828	11.128	*W*-value: 118	*W*-value: 174
	Mean	19.011	16.565	18.118	*Z*-value: –2.9035	*Z*-value: –2.3095
	Maximum	24.962	24.929	24.747	*P*-value: 0.00374*	*P*-value: 0.02088*
Expectation value (E)	Minimum	3.500	4.000	4.000	*W*-value: 47.5	*W*-value: 103
	Mean	4.787	4.714	4.760	Z-value: –1.9115	*Z*-value: –1.0645
	Maximum	5.000	5.000	5.000	*P*-value: 0.05614*	*P*-value: 0.28914*
Number of miRNA hits	Minimum	1.000	1.000	1.000	*W*-value: 30**	*W*-value: 39**
	Mean	2.785	2.285	0.678	*Z*-value: 2.2012**	*Z*-value: 2.0251**
	Maximum	33.000	35.000	05.00	*P*-value: 0.0278**	*P*-value: 0.04236**

W, *Z* values are the result of pairwise Wilcoxon *t*-test of negative control sequences when analysed against the values of plant microRNAs versus begomovirus screening.
*Not significant at $P \leq 0.05$; **Significant at $P \leq 0.05$.

followed by AC3 (miR 172 family from cca, vmu, vun; ath-miR822, hme-miR-3338, cme-miR858), BC1 (cca-miR1507a; mtr-miR5227;hme-miR-3338;cme-miR858 and gma-MiRs), AV1 (zma-miR167f-3p; osa-miR1849; cca-miR6108c) and BV1(mtr-miR2088 and sbi-miR6232). Here again soybean-derived miRNAs (gma-miR5785; gma-miR9734 and gma-miR9742) were found to target viral movement protein (ORF BC1) (Figure 2). However, the miRNAs from natural hosts like *Cajanus, V. mungo*, and *V. unguiculata* showed 22 sRNAs targeting ORFs AC1 through AC4 (including the miR 394 family from cca and vmu). Eventhough all the MYMIV-encoded ORFs are targeted at least by one plant sRNA, two MYMV ORFs (AV2 and AC5) displayed little vulnerability for any of the known plant miRNAs (Figure 2).

Conserved plant miRNAs have been shown to play a vital role in host defence mechanism[12,13]. A scrutiny of plant-conserved miRNA families targeting the viral genome revealed that miRNAs derived from *Cajanus cajan*, *G. max* and *Lotus japonicum* (Figure 1 *a*) and miRNAs from *C. cajan*, *G. soja*, *P. acconitifolius*, *P. vulgaris*, *V. unguiculata*, *Pinus densata*, *V. mungo* and *Zea mays* (Figure 1 *b*) showed cross-silencing activity with the begomovirus genome. Thus the inherent suppressing potential form various plant miRNAome vis-à-vis viral transcriptome differs considerably. Conserved plant miRNAs with antiviral potential form suitable backbone structures for use in artificial miRNAs-based resistance.

To validate our hypothesis that plant miRNAs preferentially target viral transcripts, analysis was performed with negative control as target sequences. Expectation value and free-folding energy test values from genomic components of MYMIV and MYMV species were evaluated against those obtained for animal viral genome (Table 2). The Wilcoxon *t*-test statistical analysis revealed that the algorithm parameters obtained in begomovirus targeting were not statistically significant from Parvovirus analysis (Table 2). Hence, the miRNA target analysis studies of the two geminiviruses and animal-infecting ssDNA viruses were found to be comparable. However, comparison of the number of miRNA hits from plant miRNAs versus begomovirus and that of plant miRNAs versus Parvovirus showed statistically significant Wilcoxon *t*-test statistic values (*W*, *Z* and *P*) at $P \leq 0.05$ (Table 2). This suggest that the miRNA hits on the viral genome are significant and are not an outcome of fortuitous pairing between plant miRNAs and target viruses.

In silico analysis has been employed to identify the role of miRNA-based antiviral immunity in plants[12,13]. We used soybean and two geminiviruses that infect this important crop to identify putative plant miRNAs with antiviral potential. The predicted pairings in our study suggested that the positive miRNAs could have biological antiviral function *in vivo*. The putative antiviral soybean miRNAs were recognized to modulate the expression of plant defence responsive genes, and regulators of transcriptional factors (TFs) among others[14] (gma-miR5764, gma-miR5785, gma-miR2109-regulators of NBS-LRR genes; gma-miR396g, gma-miR5374-regulators of TFs; gma-miR5668, gma-miR9734, gma-miR9742, gma-miR5674-miRNAs in seeds and cotyledons). Our findings show that miRNAs involved in stress mechanism display a binding capacity with the viral genome and encoded ORFs, suggesting the existence of molecular crosstalk between biotic and abiotic stresses at the miRNAome level.

Analysis of viral ORFs and plant miRNA-based targeting reveals that some genes of viral genome origin (AV2 and AC5 encoded by MYMV) are not targeted by any of the plant-derived sRNAs. It is premature to conclude that viruses have evolved to keep these viral genes away from plant miRNAs. *G. max* miRNAs have also shown no binding ability to the MYMIV and MYMV encoded

ORFs, namely AC1, AC2, AC3 and AC4; however, ORFs coding for MP and NSP are potential targets. Interestingly, the highest matching percentage of miRNAs against viral genomes was exhibited by *Phaseolous acconitifolius* (1.65) followed by *G. soja* (1.46) and *G. max* (1.00), which are natural hosts of begomoviruses. On the other hand, prospective miRNAome identification in legumes, where begomovirus infections are common, could provide useful and important information on the nature and identity of legume-specific miRNAs with antiviral potential.

The predicted miRNA : target pair is significant because, plants with impaired miRNA biogenesis exhibit increased susceptibility to viral infections[15]; *in planta* expression of miRNA effector molecules and absence of VSPs could potentially result in virus suppression. The putative miRNAs identified in this study are potential effector molecules, as the precursor miRNAs (pre-miRNAs) are appropriate backbone structures for carrying a miRNAs against begomovirus transcripts. Moreover, miRNA-mediated viral gene suppression is a preferred approach due to tissue-specific gene silencing, and it precludes biosafety issues such as viral gene recombination, trans-encapsidation, and off-targets associated with viral genome-derived larger transgenes.

Despite what appears to be selective targeting of the genomic regions of MYMIV and MYMV by soybean miRNAs, begomoviruses have successfully evolved to escape the host antiviral defence and continue to cause economic losses to several legume crops. Therefore, it is more likely that viral suppressors could effectively undermine the host miRNAs to mount viral counter-defence. Also, the present findings provide an alternative perspective of host–virus interactions: the putative antiviral miRNAs, by not targeting all the viral ORFs, might help the pathogen to co-exist with the host so as to establish persistent infection as in animal virus–host interactions[16]. This view is open for experimental studies considering the limited understanding of the host–virus interactions in the wake of sRNA-mediated gene regulatory networks.

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