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SHORT COMMUNICATION

Exogenous dsRNA-mediated field protection against *Pigeonpea sterility mosaic emaravirus*

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Abstract

Pigeonpea sterility mosaic emaraviruses (PPSMVs) cause sterility mosaic disease in pigeonpea which significantly reduce the crop yield. Currently there are no effective management strategies available for the control of these emaraviruses or their vector eriophyid mites. Here, for the first time, we demonstrate double stranded RNA based control of PPSMV in an open field experiment. Three genes of PPSMV, namely, the RNA dependent RNA polymerase, nucleo-capsid protein and movement protein were targeted by dsRNA-based strategy. DsRNAs for these genes were successfully produced in vitro and exogenously applied on pigeonpea plants in the field providing protection against PPSMV. Of these, the dsRNAs targeting RNA dependent RNA polymerase and movement protein gave relatively better protection when compared to the dsRNAs targeting nucleo-capsid protein. This is the first demonstration of dsRNA-mediated protection against a negative sense plant RNA virus.

Keywords Pigeonpea · *Emaravirus* · PPSMV · dsRNA · RNAi · Virus resistance

Abbreviations

dsRNA	Double stranded RNA
MP	Movement Protein
NP	Nucleocapsid Protein
PPSMV	*Pigeonpea sterility mosaic emaravirus*
PPSMV-1	*Pigeonpea sterility mosaic emaravirus* 1
PPSMV-2	*Pigeonpea sterility mosaic emaravirus* 2
RdRp	RNA dependent RNA polymerase
RNAi	RNA interference
siRNA	Small interfering RNA
SMD	Sterility mosaic disease

Sterility mosaic disease (SMD) of pigeonpea is one of the major constraints for cultivation of pigeonpea in India (Patil and Kumar 2015, 2017) . SMD is also known to occur in other South-East Asian countries, such as Nepal, Bangladesh and Myanmar (Jones et al. 2004). Two distinct emaravirus species, *Pigeonpea sterility mosaic emaravirus 1* (PPSMV-1) and *Pigeonpea sterility mosaic emaravirus 2* (PPSMV-2) are associated with sterility mosaic disease (SMD) of pigeonpea (Patil et al. 2017). Both PPSMV-1 and PPSMV-2 were shown to be present across India and exhibit sequence variability (Patil et al. 2017). The genus *Emaravirus* (family *Fimoviridae*, order *Bunyavirales*) consists of about 15 distinct species that infect pigeonpea, alfalfa, perilla, wheat, corn and other flower and fruit trees (Mielke-Ehret and Mühlbach 2012; Tatineni et al. 2014). Emaraviruses are present as double membrane-bound bodies (DMBs) in the cytoplasm of the virus-infected cells and are transmitted to plants by eriophyid mite (*Aceria cajani*) vectors (Kormelink et al. 2011; Kulkarni et al. 2002). The genome of PPSMV-1 and -2 consist of six RNA segments that are linear, negative-sense and single-stranded (Patil et al. 2017). The largest of all is referred to as RNA1, with a length of 7022 nucleotides, encoding the RNA-dependent RNA polymerase (RdRp, 2295 amino acids). The other four segments are referred to as RNA2 (2223 nt) encoding the glycoprotein (GP); RNA3 (1442 nt) encoding the nucleocapsid protein (NP); RNA4 (1563 nt) encoding the putative movement protein p4 (MP); RNA5 (1689 nt) encoding the p5 (474 amino acids)

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Table 1 Primer names and their sequences used in this study

Primer Name	Primer Sequence (5′ to 3′)	Target gene/ sequence
Primers used for cloning of PPSMV-1 sequences in pGemT easy vector		
PPSMV1-1-aSF	GCTCTAGATTGTCGACTACAATAGTTGN	RNA-1 (3989–4008 nt)
PPSMV1-1-aSR	CCCAAGCTTCCCTTGTTTAGACTAATGAN	RNA-1 (4574–4593 nt)
PPSMV1-3-aSF	GCTCTAGAATGTTCTGTCATTCAATAAAN	RNA-3 (503–523 nt)
PPSMV1-3-aSR	CGGGATCCTATATTTTTAATTAAACACACN	RNA-3 (1096–1117 nt)
PPSMV1-4-aSF	GCTCTAGAAGTCTGGAAGAATATCTTATCN	RNA-4 (212–233 nt)
PPSMV1-4-aSR	CGGGATCCAACTTTTTCATACACACCAN	RNA-4 (688–707 nt)
Primers used in first PCR amplification		
PPSMV1-RdRp-F	GGG GAT CCT TGT CGA CTA CAA TAG TTG	PPSMV1 RdRp
PPSMV1-RdRp-F	GGG GAT CCC CCT TGT TTA GAC TAA TGA	
PPSMV1-NP-F	GGG GAT CCA TGT TCT GTC ATT CAA TAA A	PPSMV1 NP
PPSMV1-NP-R	GGG GAT CCT ATA TTT TTA ATT AAA CAC AC	
PPSMV1-MP-F	GGG GAT CCA GTC TGG AAG AAT ATC TTA TC	PPSMV1 MP
PPSMV1-MP-R	GGG GAT CCA ACT TTT TCA TAC ACA CCA	
Primers used for PPSMV detection in pigeonpea plants by RT-PCR		
PPSMV1&2-F	CCTCCTAAGAGATCANTCAGN	RNA-1 (105–125 nt)
PPSMV1&2-R	ATGAATCCTGTTCATTTTCN	RNA-1 (845–826 nt)

and RNA6 (1194 nt) encoding the p6 proteins with unknown function (Elbeaino et al. 2014 2015).

Currently there are no effective strategies for the management of SMD in pigeonpea and therefore there is an urgent need of intervention employing biotechnological approaches. The discovery of RNA-interference (RNAi) has heralded a new revolution in the area of biotechnology or molecular biology (Baulcombe 1996; Waterhouse et al. 1998; Smith et al. 2000). RNAi-technology is being successfully employed for developing transgenic crop plants with desirable traits, particularly for the management of plant viral diseases (Patil et al. 2011; Patil 2018). To alleviate the concerns of environmental impact and food safety, plant biotechnologists are exploring alternative technologies that can induce robust viral resistance without engineering plants containing viral transgenic DNA.

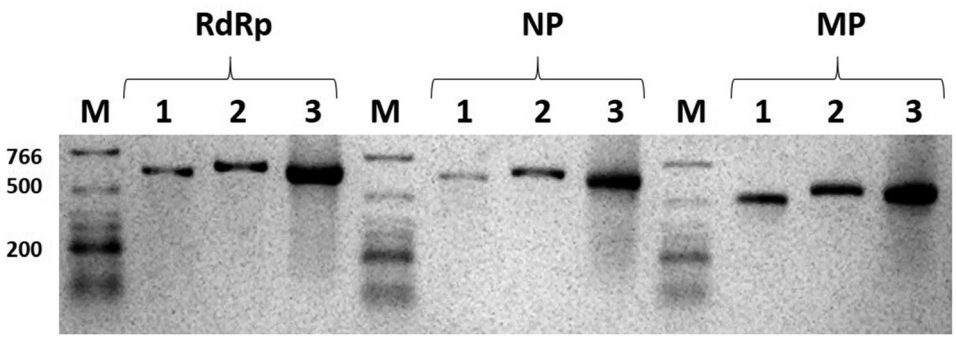

Fig. 1 Agarose gel electrophoresis of the first PCR (lanes 1) and second PCR (lanes 2) amplification products and the in vitro transcription dsRNA products (lanes 3) for the target sequences of the PPSMV-1 RdRp (606 bp), NP (621 bp) and MP (499 bp) genes of the PPSMV-1 Kalaburagi isolate. In the 1st PCR the T7 linker sequences were attached by using gene specific primers and the amplicon of this PCR was subjected to second PCR to incorporate the T7 promoter sequence, which helps in in vitro transcription of the gene sequence. M: Low Molecular Weight DNA Ladder (New England Biolabs, USA). Numbers at the left designate molecular weights in bp

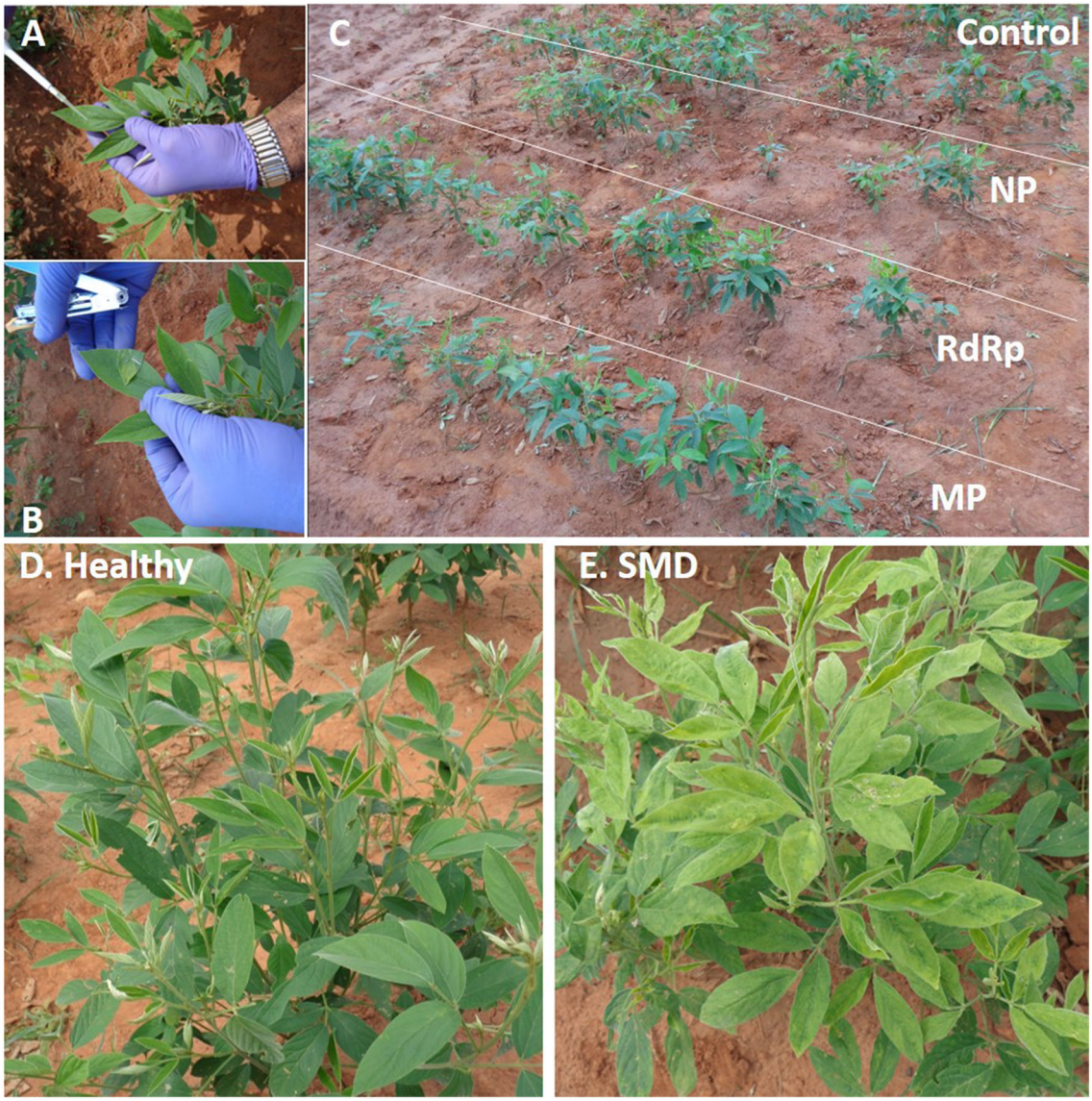

Fig. 2 Application of dsRNA on leaf surface of a healthy pigeonpea plant (**a**), followed by stapling of PPSMV infected leaf (**b**) for transmission of virus to healthy plants; One of the experimental replication with four treatments (**c**), Control, dsRNAs targeting NP (Nucleocapsid Protein), RdRp (RNA dependent RNA polymerase) and MP (Movement Protein) of PPSMV-1; A dsRNA protected healthy pigeonpea plant (**d**) and a SMD affected control pigeonpea plant (**e**)

Interestingly, in contrast to transgene derived viral resistance, RNAi can be readily induced by the topical/foliar spray application of dsRNA molecules (Tenllado and Dıaz-Ruız 2001; Yin et al. 2009; Voloudakis et al. 2018). The dsRNA molecule is central to the induction of the RNAi pathway in both transgenic and naturally virus-infected plants. Thus the progress in the area of dsRNA expression systems and the application of dsRNA as a 'spray-on' technology for non-transgenic induction of virus resistance is very important and promising for the control of plant viral diseases (Mitter et al. 2017b; Voloudakis et al. 2018). PPSMVs are also known to produce small RNAs of different size classes (Patil and Arora 2018). In this study, for the first time, we have evaluated the ability of dsRNA targeting three different genes of the emaravirus infecting pigeonpea. The target genes were RdRp, NP and MP. The conserved sequences of these three genes across different PPSMV isolates were identified and a stretch of 606 nt of RdRp, 621 nt of NP and 499 nt of MP were amplified by RT-PCR using primers specified in Table 1. The templates used were the RNA-1, RNA-3 and RNA-4 clones of PPSMV-1 Kalaburagi isolate, with GenBank accession numbers KX363886, KX363888 and KX363889, respectively (Patil and Arora 2018). These amplicons were cloned in pGem-T Easy vector from Promega (Promega, USA), using TA cloning strategy and were later sequenced to

confirm the sequences. A T7 linker sequence was added at the 5′ end of each gene specific oligo to facilitate in vitro transcription using T7 polymerase (Table1). The two-step PCR followed by in vitro transcription were done as described previously using the above PPSMV-1 clones as the template DNA (Fig. 1) (Voloudakis et al. 2015; Kaldis et al. 2018; Vadlamudi et al. 2020). The in vitro transcription was carried out using the T7 Ribomax^{TM} Express large scale RNA production system (Promega, USA) at 37 °C for 4 h, followed by 85 °C for 10 min and at 25 °C for 20 min (Vadlamudi et al. 2020). The titre of the dsRNA transcribed was estimated spectrophotometrically using Fisher Scientific Multiskan FC Reader (ThermoFisher Scientific, UK) and their quality was checked by gel electrophoresis (Fig. 1).

The dsRNA protection study was carried out in the experimental fields of AICRP on Pigeonpea, University of Agricultural Sciences, Bengaluru (Karnataka state, India), in three different replications, with 10 plants in each treatment in a random block design (Fig. 2). There were four treatments: (a) Control (No dsRNA), (b) dsRNA_RdRp, (c) dsRNA_NP, (d) dsRNA_MP of PPSMV-1. The in vitro produced dsRNA molecules targeting the RdRp, NP and MP genes of PPSMV-1 were tested for their efficacy of protection against PPSMV by exogenous application on single leaf of each pigeonpea plants. For each pigeonpea plant, 2 µl of in vitro produced dsRNA was rubbed on the leaf surface of each plant and the same leaf was subjected to PPSMV transmission by "Leaf stapling technique" (Fig. 2a, b) (Nene and Reddy 1976), using a PPSMV-1 infected pigeonpea plant as the source of inoculum. A popular pigeonpea variety "Maruthi", that is susceptible to SMD was used for the dsRNA protection experiments and the dsRNA/virus inoculations were done two weeks after the germination of the pigeonpea seeds. Symptom scoring was done using a 0–5 scale, wherein a score of "0" indicates absence of symptoms, "1" for very mild symptoms, "2" for mild, "3" for moderate, "4" for severe and "5" for very severe symptoms. The SMD symptoms were not visible up to 25 days after the transmission of PPSMV by "Leaf stapling technique"; hence all the pigeonpea plants were subjected to "nipping of the terminal bud" to enhance branching and the expression of SMD symptoms. The PPSMV infected plants exhibited chlorotic rings or mosaic symptoms on the leaves, sometimes were stunted and with partial or complete cessation of flower production. The symptoms were scored at three different intervals, i.e. 30, 35 and 40 days' post application (dpa) of dsRNA and transmission of the PPSMV.

The mean symptom scores were calculated for each treatment and replication and the data were analyzed using the statistical analysis software GraphPad Prism (Graphpad Software, USA) for all the three time points (Fig. 3). On 40 dpa, the mean symptom score for control plants without any dsRNA protection was 2.27, while it was 0.93, 1.03 and 0.66 for dsRNA-RdRp, dsRNA-NP and dsRNA-MP, respectively. The *P*-values were 0.009, 0.0039 and $<$ 0.0001 for first, second and third replications, respectively. The analysis clearly indicated a significant level of protection by PPSMV-based dsRNAs when compared to the negative control (no dsRNA), except for dsRNA-NP in the first replication. The levels of protection offered by dsRNA_MP was the highest of all the three genes, followed by dsRNA_RdRp and dsRNA_NP.

On the 40th day, randomly selected 3–4 pigeonpea plants from each of these four treatments were screened by RT-PCR using PPSMV specific primers (Table 1) to check the presence of virus in those plants. All the plants without SMD symptoms were negative for the presence of virus as screened by RT-PCR and the symptomatic plants were positive for the presence of PPSMV (Fig. 4).

With several regulatory hurdles in release of transgenic plants, exogenous application of dsRNA is a promising non-transgenic technology for control of plant viruses. Hitherto, significant number of publications have been made where plant viruses with both DNA (Namgial et al. 2019) and RNA (Tenllado et al. 2004; Gan et al. 2010; Konakalla et al. 2016, 2019; Kaldis et al. 2018; Borah et al. 2018; Vadlamudi et al. 2020), as their genetic material, have been controlled by dsRNA non-transgenic technology. However, all the plant RNA viruses that have been controlled using dsRNA-based technology are all positive sense RNA viruses, such as the papaya ring spot virus (PRSV; genus *Potyvirus*) (Konakalla et al. 2016, 2019; Kaldis et al. 2018; Borah et al. 2018; Vadlamudi et al. 2020; Worrall et al. 2019). Here for the first time, we

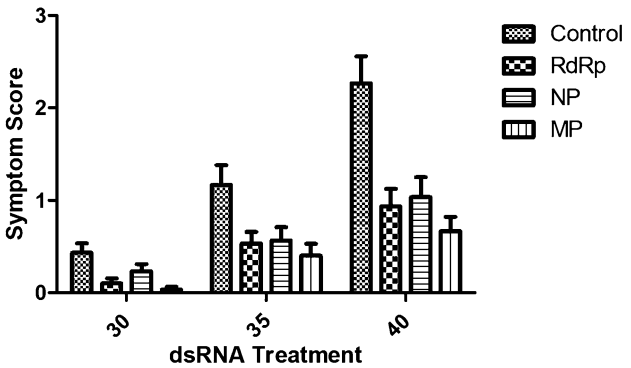

Fig. 3 Graphical representation of different levels of PPSMV infection as analysed by symptom score on 0–5 scale at three different time points for Control and dsRNAs targeting NP (Nucleocapsid Protein), RdRp (RNA dependent RNA polymerase) and MP (Movement Protein) of PPSMV-1. Symptoms were scored on 0–5 scale at 30, 35 and 40 days' post application of dsRNA and transmission of the PPSMV-1

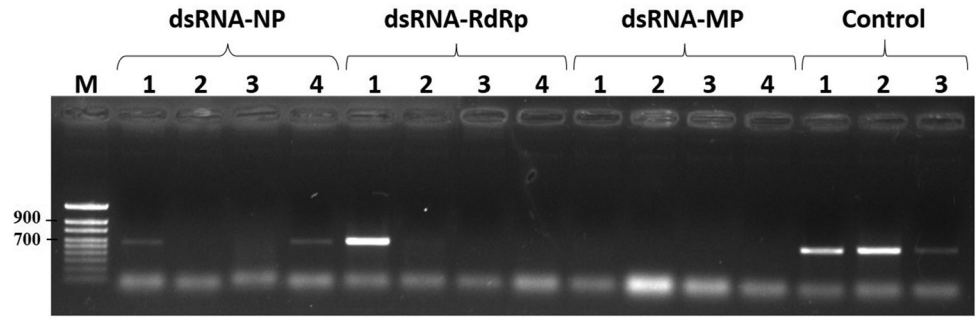

Fig. 4 Detection of PPSMV by RT-PCR using specific primers targeting the NP gene in randomly sampled pigeonpea plants treated with dsRNAs targeting NP (Nucleocapsid Protein), RdRp (RNA dependent RNA polymerase), MP (Movement Protein) of PPSMV-1 and in control pigeonpea plants. M: 100 bp DNA ladder

demonstrated at the field level that a negative sense RNA virus, such as PPSMV, can be managed by using dsRNA-based technology. Although there was no 100% protection offered by the dsRNA technology against PPSMV, reasonable level of protection was observed in the field experiments. One of the reasons for lower levels of dsRNA-based protection obtained against PPSMV-1, in contrast to the other positive sense RNA viruses, could be that there might be subsequent infection by PPSMV-2, another distinct virus that infects pigeonpea in some of the plants since it was an open field experiment. The dsRNAs targeting movement protein gave relatively better protection against PPSMV followed by RdRp when compared to levels of protection offered by dsRNA targeting Nucleocapsid protein. This apparent difference in levels of protection could be because of cell-to-cell movement and silencing suppression roles played by the movement protein (Patil and Kumar 2015). This warrants for future experiments wherein a cocktail of dsRNAs targeting the genes of both PPSMV-1 and PPSMV-2 can be evaluated to investigate a broad spectrum protection against both the emaravirus species infecting pigeonpea. Further use of nanoparticles to increase the stability and durability of the dsRNA molecules may help in commercialising this technology for the management of emaraviruses infecting pigeonpea (Mitter et al. 2017a).

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Author contributions BLP and AV conceived, designed and supervised the study. MD generated the PPSMV-1 gene constructs in ICAR-NIPB, New Delhi, BLP synthesized the dsRNA in Agricultural University of Athens and RR preformed the PPSMV protection studies in the UAS-Bengaluru farms under the supervision of MB & BLP. BLP analyzed the data and drafted the manuscript with the inputs from other co-authors.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

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