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CONSTRUCTION AND VALIDATION OF MIR159A BASED SILENCING SUPPRESSOR GENE CONSTRUCTS FOR CONTROLLING *POTATO VIRUS Y* AND *POTATO VIRUS X*

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ABSTRACT

Potato (*Solonum tuberosum* L.) is an important food crop grown throughout the world. The crop is affected with many plant viruses. *Potato virus Y* (PVY) and *Potato virus X* (PVX) are two major viruses that infect potato in single and mixed ways causing considerable yield losses. Unavailability of resistant genes and the tetraploid nature of the crop make it difficult for breeders to generate resistance to these two viruses. siRNA based and miRNA based approaches have been attempted by researches to give resistance to these viruses. In this study, two gene constructs were made using siRNA sequences that had been selected through *insilico* analysis of two suppressor genes, HC-Pro of PVY-Del66 and *p*25 of PVX-ptDel9 strains on a miR 159a backbone. The effectiveness of these two constructs in controlling PVY and PVX were evaluated transiently through agroinfiltrations in *Nicotiana benthamiana* L. and potato through post inoculations. The same constructs were also evaluated in potato plants, which had already got natural infections of PVY and PVX viruses. Results showed that the two constructs were effective in reducing PVY and PVX virus concentrations in *N. benthamiana* and potato in post inoculations. It was also observed that the two constructs were able to control the virus load in plants that had already been infected with PVY and PVX. These two constructs can be used to generate transgenic resistance against PVY and PVX.

KEY WORDS: *Potato virus Y, Potato Virus X,* siRNA, miRNA, gene constructs

INTRODUCTION

Potato (*Solanum tuberosm* L.) is an important food crop grown throughout the world. It is a tetraploid crop with 48 chromosomes. A number of viruses infect the potato crop and *Potato virus Y* (PVY) and *Potato virus X* (PVX) are two major viruses that affect potato worldwide. Tetraploid nature of the crop makes it arduous to breed resistant varieties for these viruses. Pathogen derived resistance is one of the important aspects, which is used to generate resistance against virus diseases in the absence of natural resistance.

Some plant use RNA silencing mechanism, which is also known as cosuppression or RNA mediated virus resistance or gene silencing to get protected from virus infections (Baulcombe, 1996; Chellappan *et al.*, 2004). RNA silencing was initially reported from *Caenorhabditis elegans* (Fire *et al.*, in 1998). In RNA silencing against plant viruses, double stranded RNA (dsRNA) of plant viruses generated during replication or by the action of plant RNA dependent RNA polymerase (RdRp) are cleaved by dsRNA-specific RNAs III (dicer), yielding 21-26 long short interfering RNAs known as siRNAs (Chapman *et al.*, 2004). Next, those siRNAs are recruited to an RNA induced silencing complex (RISC) where mRNAs are degraded in a sequence specific manner. Proteins of Argonaute family, nucleases and other factors also involve in RISC. These small RNA molecules can also suppress the protein translation (Voinnet, 2001). Small interfering RNAs (siRNAs) can be of endogenous origin (miRNAs) or exogenous origin (siRNA). siRNAs are generally triggered by transgenes or invading plant viruses whereas miRNAs are of natural origin in eukaryotes. miRNA are processed from imperfect hairpin forming RNA precursors transcribed from miR genes in the same way by Dicer like proteins as siRNAs (Ambros *et al.*, 2003). miRNAs act as negative regulators on target mRNAs. They involve in gene silencing either through site-specific cleavage by RISCs or translational repression (Bartel, 2004). Once the RNA silencing mechanism is started by a foreign molecule it can be triggered by any other RNA species, which bare sequence homologies (Voinnet, 2001).

To counter plants' natural antiviral defense mechanism, viruses have evolved with genes encoding for proteins that suppress naturally occurring RNA

silencing mechanism in plants. Those proteins are known as silencing suppressor proteins (Baulcombe, 2001). P1/HC-Pro of potyviruses (Anandalakshmi *et al.*, 1998) and *p*25 of *Potato virus X* (Voinnet *et al.*, 2000) are two earlier identified silencing suppressors and well studied silencing suppressors.

Potyvirus HC-Pro is one of the earliest known silencing suppressors (Qu and Morris, 2005). It involves in polyprotein processing, amplification of genome, long distance movement and aphid transmission of PVY (Kasschau *et al.*, 1997) and acts as a pathogenecity enhancer causing increased accumulation of viral RNAs of several unrelated plant RNA viruses (Pruss *et al.*, 1997). Initially, *p*25 of PVX (TGBp1) was identified as a movement protein (Baynet *et al.*, 2005). It has helicase activity and interferes with plasmodesmal gating. It acts as a silencing suppressor by interfering with the mobilization of silencing signals and degrading argonaute proteins (Chiu *et al.*, 2010; Voinnet *et al.*, 2000).

dsRNA of plant viruses are the key targets of transgene mediated RNA silencing; i.e. generation of barley lines resistant to barley yellow dwarf virus-PAV (BYDV-PAV) (Wang *et al.*, 2000), tobacco lines resistant to PVY (Zhu *et al.*, 2004), Tomato yellow leaf curl complex resistance (Abhary *et al.*, 2006), potato lines resistant to PVY (Missiou *et al.*, 2004) and marker free PVY and PVX resistant potato (Bai *et al.*, 2009). Recently the antiviral strategy of artificial micro RNA (amiR) has been applied in achieving antiviral resistant transgenics for *Turnip yellow mosaic virus*, *Turnip mosaic virus*, *Cucumber mosaic virus*, *Plum Pox Virus* and PVY (Duan *et al.*, 2008; Jiang *et al.*, 2011; Niu *et al.*, 2006; Qu *et al.*, 2007; Simón-Mateo *et al.*, 2006). In this study miR159a based two artificial constructs were made based on two viral suppressor genes, PVY-HC-Pro and PVX-*p*25 and their performance against two potato virus isolates, PVY Del66 and PVX ptDel9 were evaluated in *Nicotiana benthamiana* L. and potato.

MATERIALS AND METHODS

Amplification of PVY-HC-Pro and PVX-*p*25 and selection of siRNA

Total plant RNA was extracted from *N. benthamiana* plants 14 days post

inoculation with PVY Del66 and PVX ptDel9 strains, which were maintained in the glass house of the Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi, India using TRIzole (Life Technologies, Grand Island, NY, USA). cDNA was synthesized from 50 ng of total plant RNA, using RevertAid First Strand cDNA Synthesis Kit (Fisher Scientific-USA, Pittsburgh PA, USA) according to the manufacturer's protocol. The primers BM 270F; 5′GGGGTTATGGATTC AATGGTTC3′, BM271R; 5′ACCAACTCTATAATGCTTAATG3′ and BM296F; 5′ATGGATATTCTCATCATTAG3′, BM 297R; 5′CTATGTCCCTGCGCGG3′, were designed (synthesized at Sigma Aldrich Co. India) and used to amplify PVY-HC-Pro (1395 bp) and PVX-*p*25 (681 bp) portions, respectively. cDNA synthesis and polymerase chain reaction (PCR) were carried out in a MJ-Mini Personnel Thermal Cycler Model PTC-1148C (Bio-Rad Laboratories, Hercules, CA, USA). In each amplification, the PCR mixture of 25 µl consisted of 50 ng of cDNA, 0.5 µl of 10 mM forward and 10 mM reverse primers each, 0.5 µl of *Taq* DNA Polymerase (5units/µl) (RBC Bio Science Corporation, New Taipei City, Taiwan), 1.0 µl of 10 mM dNTP mix, 2.5 µl of 10X PCR buffer (RBC Bio Science Corporation, New Taipei City, Taiwan) and sterile distil water. PCR cycles consisted of initial denaturation at 94 °C for 2 min. followed by a 30 cycles of denaturation at 94 °C for 2 min, annealing at 58 °C for 40 sec, and extension at 72 °C for 30 Sec. The final extension was carried out at 72 °C for 7 minutes. The amplified products, PVY-HC-Pro and PVX-*p*25 and were ligated to RBC TA Vector (RBC Bio Science Corporation, New Taipei City, Taiwan) as per the manufacturer's protocol. Plasmid isolation was carried out using mdi pDNA miniprep kit (mdi Membrane Technologies LLC, CA, USA) as per the manufacturer's protocol. Sequencing of DNA inserts were carried out in a high-throughput sequencer; ABI 3730 XL at Xcelris laboratory, Bangalore, India (www.xcelrislabs.com). The sequence data of PVY-HC-Pro and PVX-*p*25 were analyzed using Ambion siRNA target finder (http://www.ambion.com/ techlib/misc/siRNA_finder.html). Generated siRNA sequences were analyzed using NCBI local blast (http://blast.ncbi.nlm.nih.gov) and the best suited two siRNA portions were selected from HC-Pro and *p*25 sequences, separately based on targets matching.

Designing of miR159 precursor

Total plant RNA was isolated from leaves of *Arabidopsis thaliana* using

TRIzole (Life Technologies, Grand Island, NY, USA). cDNA was synthesized from total plant RNA using RevertAid First Strand cDNA Synthesis Kit (Fisher Scientific-USA, Pittsburgh PA, USA) according to the manufacturer's protocol. miR159a precursor was then synthesized based on this cDNA using miR159a precursor primers; (BM341F: 5′CACCACAGTTTGCTTATGTCGGATCC3′ and BM342R: 5′TGAGTCGACA TGTAGAGCTCCCTTCAATCC3') described by Niu *et al.* (2006). (The primers were synthesized at Sigma Aldrich Co. India). cDNA synthesis and follow up PCRs were carried out in a MJ-Mini Personnel Thermal Cycler Model PTC-1148C (Bio-Rad Laboratories, Hercules, CA, USA). The PCR reaction mixture consisted of 50 ng of cDNA as template, 5 µl of 10Xreaction buffer, 4 µl of 2.5mMdNTP mix, 2 µl each of forward and reverse primers (10 picomole), 1.25 U of Ex Taq DNA polymerase (Takara, Japan) and autoclaved water to makeup 50 µl of total volume. The PCR cycle consisted of initial denaturation of 98 °C for 30 seconds followed by 40 PCR cycles, each consisting of denaturation at 98 °C for 10 seconds, primer annealing at 57 °C for 45 seconds, and extension at 72 °C for 1 minute. The final extension was done at 72 °C for 10 minutes. The amplified product was purified using Nucleospin gel and PCR clean-up kit (Macherey-Nagel Inc., PA, USA) as per manufacturer's protocol and ligated to RBC TA Vector (RBC Bio Science Corporation, New Taipei City, Taiwan) according to the manufacturer's protocol. Sequencing was done in a high-throughput sequencer; ABI 3730 XL at Xcelris laboratory, Bangalore, India (www.xcelrislabs.com).

Preparation of miR constructs (amiR159a)

The primers indicated in the Table 1 were designed (synthesized at Sigma Aldrich Co. India) and used to amplify HC-PromiR and *p*25miR constructs replacing the naturally occurring siRNA portions of the miR159a backbone with already identified siRNA portions from PVY-HC-Pro and PVX-*p*25 gene sequences. Restriction sites; *Kpn*1 at 5′and Sal1 at 3′ were also incorporated along with primers for downstream ligation to pBinAR vector. PCRs were carried out in a MJ-Mini Personnel Thermal Cycler (Model PTC-1148C, Bio-Rad Laboratories, Hercules, CA, USA).

The PCR reaction mixture consisted of 50 ng of RBC TA vector containing miR159a backbone as the template, 5 µl of 10Xreaction buffer, 4 µl of 2.5mMdNTP mix, 2 µl each of forward and reverse primers (10 picomole), 1.25 U of Ex *Taq* DNA polymerase (Takara, Japan) and autoclaved water to makeup 50 µl of total volume.

Table 1. The primers designed for developing artificial micro RNA constructs based on PVY HC-Pro and PVX *p*25 genes

Primer Name	Primer sequence (5′ to 3′)	Annealing Temperature (°C)	Construct name	Amplicon size (bp)
BM361F	caggtacctgatctgacgatggaagaaacgcag acatgccatgtggcatgagttgagcagggta	64	HC-PromiR	219
BM362R	tgagtcgacatgaaacgcagacatgccatgtgg gaagagtaaaagccatta			
BM 367F	caggtacctgatctgacgatggaagaatagaggaaat tggccagtccatgagttgagcagggta	64	*p*25miR	219
BM368R	tgagtcgacatgaatagaggaaattggccagt cgaagagtaaaagccatta			

The PCR cycle consisted of an initial denaturation at 98 °C for 30 seconds followed by 40 PCR cycles, each consisting of denaturation at 98 °C for 10 seconds, primer annealing at 64 °C for 45 seconds, and extension at 72 °C for 1 minute. The final extension was done at 72 °C for 10 minutes. The amplified products were purified using Nucleospin gel and PCR clean up kit (Macherey-Nagel Inc., Bethlehem, PA, USA) as per manufacturer's protocol and ligated to RBC TA Vector (RBC Bio Science Corporation, New Taipei City, Taiwan) according to the manufacturer's protocol. Sequencing was done in high-throughput sequencer; ABI 3730 XL at Xcelris laboratory, Bangalore, India (www.xcelrislabs.com). The constructs were named as HC-PromiR and *p*25miR

Transformation of constructs to binary vector

miR159a constructs were released from TA vector using endonucleases,

*Kpn*1 and *Sal*1. Then the constructs were purified from 1% Agarose gel using Nucleospin gel and PCR clean up kit (Macherey-Nagel Inc., Bethlehem, PA, USA) as per manufacturer's protocol and ligated to pBinAR vector (~12kb) that had already been restricted with *Kpn*1 and *Sal*1 endonucleases. The ligation reaction was carried out using Fermentas T4DNA ligase and ligase buffers (Fermentas, Glen Burnie, MD, USA). The ligation reaction consisted of 3 µl of 50 ng DNA insert, 1 µl of 50 ng vector, 1 µl of T4DNA ligase and 2 µl of 10X ligase buffer. Ligated vectors were transformed to *Escherichia coli* DH5α competent cells. The plasmids were isolated using mdi pDNA miniprep kit (mdi Membrane Technologies LLC CA, USA) as per the manufacturer's protocol and the inserts were confirmed through PCR.

Transient assays

Agroinfiltration studies with *Nicotiana benthamiana*

pBinAR plasmids carrying HC-PromiR and *p*25miR constructs were transformed to *Agrobacterium tumefaciencs* (strain: EHA 105) with helper component PRK2013 using triparental mating method as described by Goldberg and Ohman (1984). *N. benthamiana* plants, which were negative to PVY and PVX in Direct Antigen Coating-Enzyme Linked Immunosorbent Assay (DAC-ELISA) (Clark and Adams, 1977) were selected as healthy plants for the assay. The Agroinfiltration was carried out in *N. benthamiana* as per the method described by de Felippes and Weigel (2010). Well expanded three to four leaves of each plant were used for agroinfiltration under each treatment. Only miR construct and pBinAR were also transferred to plants through agroinfiltration in the same way as checks. Three days post agroinfiltration (dpa) the same leaves were mechanically inoculated with PVY Del66 and PVX ptDel9 strains, separately. The inoculum was prepared by grinding 100 mg of freshly harvested infected (PVY/PVX) leaf tissues in 1 ml of 0.1 M phosphate buffer, pH 7.0 containing 0.2% sodium sulfite and 0.01 M 2-mercaptoethanol using chilled mortars and pestles. The extract was filtered using cheesecloth. One per cent of Celite 545 (S.D. Fine Chem. Ltd., Hyderabad, India) was added to the inoculum. Leaves to be inoculated were predusted with carborundum (600 mesh) (HiMedia Laboratories, Mumbai, India). Inoculation was done by rubbing leaves with fore finger that had been

wetted in the inoculum. Symptom development was observed at the whole plant level at 14 days post inoculation (dpi). PVY and PVX virus concentrations were also measured at 14 dpi through Enzyme Linked Immunosorbent Assay (ELISA) using PVY and PVX specific polyclonal antibodies. Absorbance was measured at 405 nm in an ELISA reader (BIOTEK Instruments, USA)

Agroinfiltration studies with PVY and PVX infected potato

Four to six week old potato plants (5 plants for each treatment), which had already been identified using ELISA for the presence of PVY, PVX and double infections were agro infiltrated with HC-PromiR and *p*25miR constructs in the same way as described in the previous section for *N. benthamiana*. Virus concentrations of agro infiltrated leaves were quantified using ELISA as described above at 14 dpi.

Agroinfiltration studies with healthy potato followed by challenge inoculations

In this experiment field potato plants identified as healthy through DAC-ELISA using PVY and PVX specific antibodies were agro infiltrated with HC-PromiR and *p*25miR constructs and challenge inoculated 03 days post agroinfiltration (dpa) as follows. HC-PromiR in 12 plants followed by PVY inocultaion, *p*25miR in 8 plants followed by PVX inoculation and PVY HC-Pro and PVX-*p*25 together in 8 plants followed by PVY and PVX mixed inoculations. In each case positive controls (only the respective virus inoculation) and negative controls (healthy) were maintained. Virus concentrations of treated leaves were quantified at 14 dpi using DAC-ELISA as described previously.

Confirmation of HC-PromiR and p25miR constructs in agro infiltrated leaves

Total leaf DNA was isolated from agro infiltrated leaf samples using DNAeasy plant minikit (Qiagen, Valencia, CA, USA) 07 dpa. The presence of constructs were confirmed with construct specific primers in PCR.

RESULTS AND DISCUSSION

Amplification of miR159a precursor, PVY-HC-Pro and PVX-*p*25 genes

BM 341 and BM 342 primers amplified 277 nt miR159a precursor (Figure 1a). BM 270F and BM 271 R primers amplified a 1395 bp portion for PVY-HC-Pro and BM 296 F and BM 297 R a 681 bp portion for PVX-*p*25. (Figure 1b and 1c).

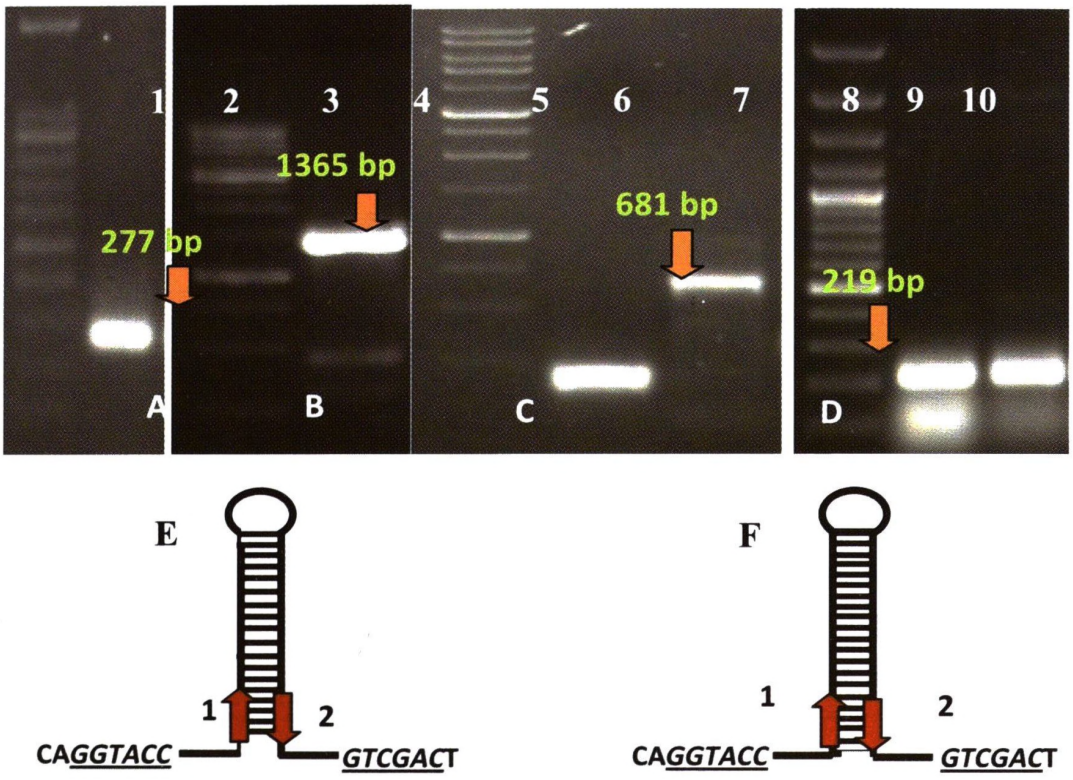

Figure 1. Amplification of precursor, suppressor genes, gene constructs
Note: (A). miR159a precursor (277 bp), (B) *Potato virus Y*-HC-Pro (1365 bp) from PVY Del66 strain, (C) *Potato virus X-p*25 (681 bp) from PVX ptDel9 strain. (D) amiR159a with HC-Pro and *p*25 siRNA sites, (E) HC-PromiR construct, (F) p25miR construct. Lane 1 = 100 bp ladder, Lane 2 = miR159a precursor, Lane 3 = 1KB ladder, Lane 4 = PVY-HC-Pro, Lane 5 = 1kb ladder, Lane 6 = PVX-p8 (an internal control) Lane 7 = PVX-*p*25. Lane 8 = 100 bp ladder, Lane 9 = PVY-HC-Pro, Lane 10 = PVX-*p*25, Underlined sequences are restriction sites for *Kpn*1 and *Sal*1.

Selection of siRNA and development of HC-PromiR and *p*25miR constructs

Best suited siRNA sequences selected from the Ambion siRNA target

finder, which were subsequently used in HC-PromiR and *p*25 miR construct preparations are indicated in the Table 2. BM 361F, BM 362R primers amplified HC-PromiR (219 bp) fragment and BM 367F, BM 368R primers *p*25miR (219 bp) from the TA vector containing miR159a backbone (Figure 1d). Sketches of the precursors of HC-PromiR and *p*25miR are indicted in the Figure 1e and 1f.

Table 2. Selected siRNA sequences form *Potato virus Y*-HC-Pro and *Potato virus X*-*p*25 sequences that were used to prepare artificial micro RNA constructs

Gene	Targeting sequence (5′ to 3′)	Sense strand (5′ to 3′)	Antisense strand (3′ to 5′)
HC-Pro	aaacgcagacatgccatgtgg	acgcagacaugccauguggtt	ttugcgucuguacgguacacc
*p*25	aauagaggaaauuggccaguc	uagaggaaauuggccaguctt	ttaucuccuuuaaccggucag

Confirmation of HC-PromiR and *p*25miR constructs

PCR with primers BM361F, BM362R and BM 367F, BM 368R confirmed the presence of HC-PromiR and *p*25miR in agro infiltrated leaves of *N. benthamiana* (Figure 2 A).

Symptom expression and coat protein levels in *Nicotiana benthamiana* with HC-PromiR and *p*25miR

All the inoculated (+ ve control) plants showed symptoms at 14 dpi. However, no visual symptoms were observed in *N. benthamiana* plants, in which HC-PromiR and *p*25miR had been kept and challenge inoculated with PVY-Del66 and PVX-ptDel9 isolates, respectively at 14 dpi. Results revealed that the constructs: HC-PromiR + PVY, *p*25miR+PVX had a good control of respective viruses at 14dpi (Table 3, Figure 2 B).

Symptom expression and coat protein levels in PVY and PVX infected potato after the agroinfiltrations

A reduction in symptoms in the newly appearing leaves was observed in infected potato plants, which had been agroinfiltrated with HC-PromiR and *p*25miR constructs at 14 dpi. ELISA absorbance values showed a considerable reduction in the virus concentration of PVY and PVX in PVY, PVX and PVY+PVX infected plants, which were agro infiltrated with HC-PromiR, *p*25miR singly and doubly, respectively (Table 4).

Table 3. Symptom development in agro infiltrated *N. benthamiana* plants at 14 days post inoculation

Construct	Challenge inoculation	Symptomatic plants out of 5 inoculated (numbers)	Symptoms
Healthy	PVY	5	Yellow spots, Puckering
HC-PromiR	PVY	0	No symptoms
HC-PromiR	No inoculation	0	No symptoms
Healthy	PVX	5	Yellow mottling
*p*25miR	PVX	0	No symptoms
*p*25miR	No inoculation	0	No symptoms
miR159a	No inoculation	0	No symptoms
pBinAR	No inoculation	0	No symptoms
Healthy	No inoculation	0	No symptoms

Note: [x] Infection was confirmed by ELISA

Response of HC-PromiR and *p*25miR agro infiltrated potato plants to PVY and PVX post inoculations

Significant reductions in PVY and PVX contents of HC-PromiR and *p*25miR agro infiltrated and followed by challenge inoculated leaves when compared to none agro infiltrated followed by challenge inoculated leaves were observed. This was also evident in the leaves, which were agro infiltrated with both the constructs followed by PVY and PVX duel inoculation (Figure 3 B and 3 C).

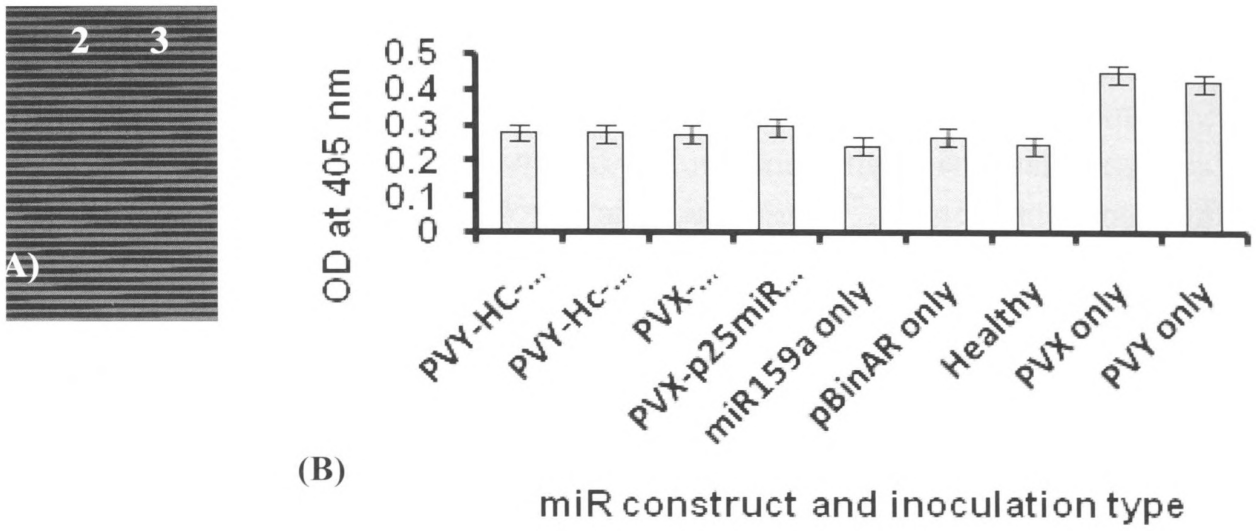

Figure 2. Confirmation of HC-PromiR and *p*25miR constructs in *N. benthamiana* (A) and Detection of virus concentrations in *Nicotiana benthamiana* leaf tissues agro infiltrated with HC-PromiR and *p*25miR constructs and challenge inoculated with respective viruses and in control treatments (B)'.Note: Lane 1 = 1kb ladder, Lane 2 = PVY-HC-PromiR, Lane 3 = PVX-*p*25miR,

Table 4. Symptoms and ELISA values of *Potato virus Y* and *Potato virus X* infected plant leaf tissues, agro infiltrated with HC-PromiR and *p*25miR constructs

Virus present	Construct	Before the agroinfiltration with construct		14 days after the challenge inoculation	
		Symptoms	OD at 405 nm	Symptoms	OD at 405 nm
Healthy	No construct	No symptoms	0.068	No symptoms	0.120
PVY	No construct	Curling, puckering of leaves, stunting	0.300	Curling, puckering of leaves, stunting	1.12
PVY	HC-PromiR	Curling, puckering of leaves, stunting	0.300	New leaves without symptoms	0.209
Healthy	No construct	No symptoms	0.056	No symptoms	0.041
PVX	No construct	Leaf necrosis	0.507	Leaf necrosis	1.100
PVX	*p*25miR	Leaf necrosis	0.507	New leaves without symptoms	0.229

Unavailability of suitable resistance genes and high ploidy level of crops such as potato make it difficult for breeders to generate resistance for plant pathogens. In this circumstance, pathogen derived resistance along with transgenic approaches are important in managing plant pathogens. siRNA based and miR based transgenic silencing approaches have been tried worldwide to tackle various plant invading viruses. Vaucheret *et al.* (2004) reported that some nucleotides of mature miR sequences of plants can be replaced with some other nucleotides without compromising the biogenesis of miR. Such modified miRNAs are known as artificial microRNAs (amiR) and they can be used to target viruses. In this study the same concept was employed. Many investigators have attempted to improve the resistance efficiency through choice of preponderant precursor backbones, expression of a dimeric amiRNA precursors/amiRNAs (Ai *et al.*, 2011; Niu *et al.*, 2006). Niu and co-workers in 2006 made the first attempt with miR constructs for *Turnip yellow mosaic virus*.

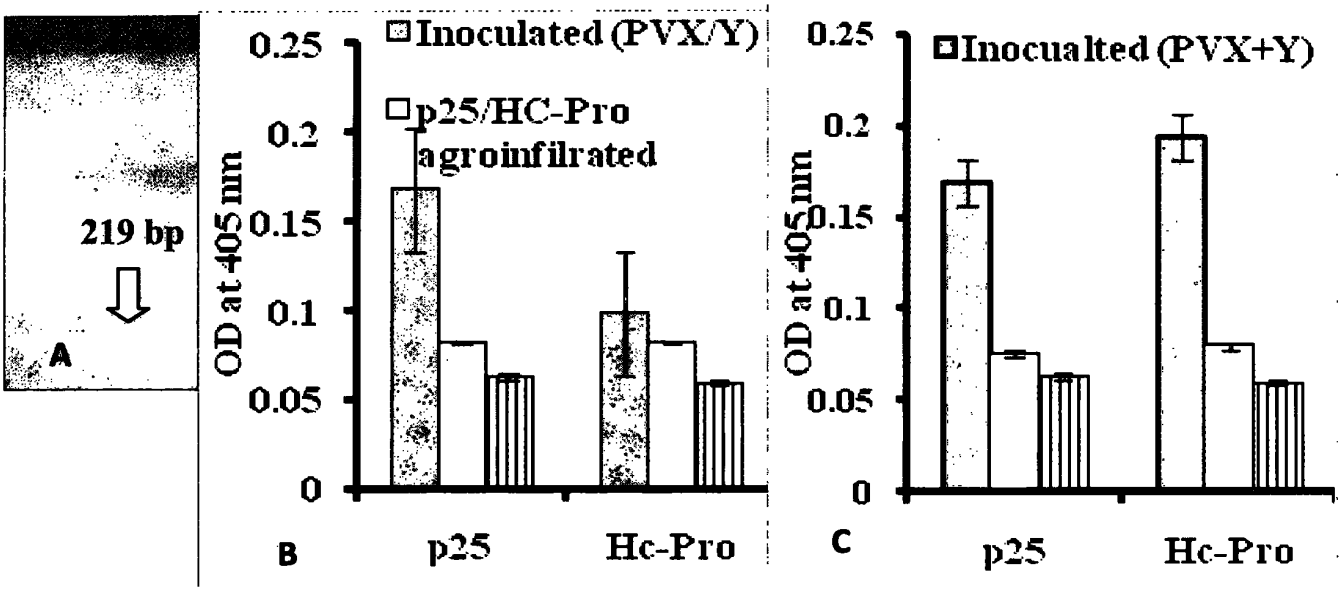

Figure 3. Confirmation of HC-PromiR and *p*25miR constructs in potato leaves (A), Average level of virus concentrations in agro infiltrated potato leaves (either with HC-PromiR or *p*25miR), which were post inoculated with PVY/PVX (B), Average level of virus concentrations in agro infiltrated potato leaves (mixed with HC-PromiR and *p*25miR), which were post inoculated with PVY+PVX (C)

Note: Lane 1 = 1kb ladder, Lane 2 = PVY-HC-PromiR, Lane 3 = PVX-*p*25miR, B & C. Detection of virus concentrations in potato leaves (14dpi), first agro infiltrated with HC-PromiR and *p*25miR constructs followed by challenge inoculated with PVY and PVX singly and doubly. Healthy = Healthy, uninoculated

Qu *et al.* in 2007 showed that the expression of miR was more effective than siRNA in inhibiting virus suppression in *Cucumber mosaic virus*. Jiang and co-workers (2011) designed an artificial microRNA (amiRcp-8) targeting the 30 end region of PVY-CP, which induced a very high virus resistance and they further demonstrated that transgenic plants carrying pre amiRNAs can effectively express 21 nt, mature, functional amiRNAs, which confer a good virus resistance. Zhang *et al.* (2011) showed that artificial micro RNA can generate stable resistance against CMV in tomato. In this study, a similar approach was made.

The results were promising in controlling the virus infection and a significant reduction in the concentrations of PVY and PVX viruses were observed with HC-PromiR and *p*25miR in transient assays with *N. benthamiana* and potato.

Jiang *et al.* (2011) stressed that the siRNA target selection is crucial for achieving a successful virus control in transgenic plants and the best results to avoid the microheterogenecity nature of the pathogen can be achieved selecting highly conserved regions of the sequences. In this study siRNA sequences were carefully selected not only from highly conserved regions but also from highly target specific sequences using *in-silico* analyses. The selected sequences did not show off target attacks and the same was evident phenotypically (data are not shown) by the observation that confirmed none availability of any malformation in the plants (data are not shown), agroinfiltrated with the HC-Pro-miR and *p*25miR in the study.

Qu *et al.* (2007) showed that *Cauliflower mosaic virus* (CaMV) 35S promoter driven miRNA precursor sequence was more effective targeting *2b* of CMV and inhibiting CMV viral activity in transient expression systems and transgenic plants of tobacco than *Pol* III promoter-driven shRNA construct. In this experiment, HC-PromiR and *p*25miR constructs were kept downstream a 35S CaMV promoter and that must have also contributed to the success of constructs in controlling virus level and reducing symptoms in target plants with pre and post infections of PVY and PVX.

When compared to long dsRNA-mediated antiviral silencing, the artificial miRNA strategy is reported with several advantages (Jiang *et al.*, 2011). First,

they are friendlier to the environment since only 21 nt portion is used, which is also in naturally occurring viral nature (Garcia and Simon-Mateo, 2006; Qu *et al.*, 2007). Second, a long viral cDNA fragment may find a greater homology with host plant genes, creating more off target effects, influencing the expression of plants native genes. In contrast, a small RNA fragment used in a miR based construct minimizes such effects (Duan *et al.*, 2008). Third, being small portions of nucleotides, multiple virus-specific miRNAs can be combined together to give protection to multiple viral infections (Niu *et al.*, 2006). The constructs used in this study is from plant origin (*Arabidopsis thaliana*), which is naturally involved in RNA silencing mechanism across a number of plant species. Therefore, these constructs are environmental friendly. The siRNA portion of each of construct was only 21 nt long. Therefore, there is less possibility for the construct to aim off targets when compared to larger gene portions used in developing transgenic resistance for plant viruses. Being plant origin and small nt portions the immunity generated by these two constructs may sustain and would not create biosafety problems in the generation of transgenic plants for commercial use. However, further studies at large scale are needed to confirm any other effect of these constructs before commercial use.

CONCLUSIONS

HC-PromiR and *p*25miR constructs are effective in controlling PVY and PVX in single and mixed infections. The constructs are friendlier to the environment since only 21 nt portion has been used and it is also in naturally occurring viral nature and they are effective in reducing PVY and PVX virus loads in already infected (single and mixed) plants. There is less possibility for the construct to aim off-targets when compared to larger gene portions used in developing transgenic resistance for plant viruses. The HC-PromiR and *p*25miR constructs can be used effectively to develop transgenic resistance for Potato virus Y and Potato virus X.

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