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Comparative Silencing Effect of Different siRNA Fragments on Potato Virus X Coat Protein in Transient Transfection Assays

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ABSTRACT

Coat protein gene of potato virus X is a structural protein that plays a vital role in viral transmission and pathogenesis. RNA interference or post transcriptional gene silencing is a regulatory conserved mechanism that uses small interfering RNAs to control the expression of desired gene by inhibiting mRNA transcription. The present study aims to investigate the potential role of different siRNAs in down-regulating the mRNA expression of potato virus X in cell culture assays. Three specific siRNAs against CP-PVX gene were designed in which siRNA 3 significantly reduced the CP-PVX mRNA expression in HepG 2 cells upto 91.91% (12.5 fold) as compared to siRNA 1 and siRNA 2 where knockdown percentages were only 20 and 73.04%, respectively as revealed by realtime PCR. Maximum knockdown values were obtained at 100 nM concentration of siRNA 1 and 2, while for siRNA 3 promising knockdown was obtained at 50 nM concentration. For long lasting effect of siRNAs, short hairpin RNA corresponding to siRNA3 was designed and tested against CP-PVX which resulted in a similar pattern of inhibition on RNA expression of target gene as synthetic siRNA3. It was found that mRNA expression of targeted gene was reduced to 91% depicting strong RNAi response generated by shRNA. Our findings support the possibility of using consensus siRNA and shRNA-based approach as a promising strategy in effective inhibition of PVX in *in-vitro* assay. Down regulation of potato virus X through coat protein gene targeted siRNA is a potential way of inhibiting invading virus and to effectively stop its systemic spread.

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AK carried out the research. FN performed transfection assays. BT planned and designed the study. AOS helped in real-time assays. KS wrote the manuscript. NS prepared figures. AMK is the advisor of FN. AMK and IAN proofread the manuscript.

Key words
Coat protein gene, HepG 2-PVX infected cells, RNA interference, Potato virus X

INTRODUCTION

Potato virus X (*PVX*) is the member of Potexvirus and a single stranded positive polarity RNA virus with genome size of 5.9 - 7.0 kb. It is the most prevalent potato virus that spread mechanically and infects solanaceous, *Chenopodiaceae, Amaranthaceae* and *Fabaceae* crops (Alhoot *et al*., 2012). Infection by *PVX* usually cause leaf molting and slight stunting, but in some cases the foliage may have distinct yellowing pattern depending upon the viral strain. A yield loss up to 10 – 50% was recorded in potato due to *PVX* alone (Bai *et al*., 2009) while mixed infection with *PVY* dramatically decrease the yield to 50 –70%.

Various antiviral strategies have been opted to create tolerance in plants against *PVX*. These include cross protection (Baulcombe, 1996), insertion of virus derived movement protein gene (Bitko *et al*., 2005), coat protein gene, replicase gene (Chapman *et al*., 1992), antisense RNA mediated resistance and virus induced gene silencing (Chen and Okayama, 1987). The resistance efficacy of all these strategies varies and scientists met with mixed success (Culver, 1996; Alba *et al*., 2002; Dalakouras *et al*., 2011). The resistance conferred by these strategies was protein mediated some and RNA mediated others. Mechanism behind the protein mediated resistance is still unclear but RNA mediated resistance is now termed as RNA interference (RNAi) which is now a powerful tool applied in functional genomics.

RNAi is a naturally conserved regulatory mechanism in eukaryotic organisms that has evolved against potential threats of viruses and transposons (Duan *et al*., 2008). It is a process of gene silencing where small interfering RNA (siRNA) triggers sequence specific degradation of cognate mRNA (Echeverri and Perrimon, 2006). siRNAs are 21 -

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25bp long dsRNA molecules that act as RNAi pathway initiators and trigger systemic gene silencing (Elbashir *et al*., 2001). Currently, diverse gene silencing approaches; like sense/antisense RNA, shRNA, small/long hairpin RNA and miRNA precursors are being used to initiate RNAi pathway (Goregaoker *et al*., 2000; Faivre-Rampant *et al*., 2004; Gao *et al*., 2013).

Studies have shown that siRNAs can elicit a potent and sequence specific response in cultured cell lines without immune response (Hamilton and Baulcombe, 1999). Today, several strategies have been used to trigger RNAi response in cultured cells to knockdown targeted genes (Hannon and Rossi, 2004). In siRNA targeting strategies, off-target gene silencing and cross-reaction have also been reported (Hinton *et al*., 2013; Jackson *et al*., 2003). Initially the non-specific nature of siRNA was credited to the sequence and thermodynamics of siRNA. Recently, with the improvement in the siRNA design algorithms, the problems associated with these factors have been minimized but still bulk of siRNAs needs to be designed to recognize most potent siRNA. In the present study, we aimed to compare the effect of siRNA and shRNA to specifically target coat protein gene of local PVX isolate.

MATERIALS AND METHODS

siRNA and shRNA oligo design and synthesis

A web-based siDESIGN algorithm was used to design potential siRNAs against CP-*PVX* gene and deduce the probable silencing efficiency. Three siRNAs were selected targeting CP-*PVX* gene at different positions. The designed oligos contain 29 bp including 8 bp of T7 promoter at 5' overhang (Table I). The designed oligo templates were custom synthesized from ThermoScientific (USA) while siRNAs were synthesized by using siRNA construction kit (Ambion, USA) as per instructions. The annealed siRNAs comprising of sense, antisense oligos were used for subsequent transfection assays. For shRNA oligo, loop sequence and 3, 5 flanking sequences were taken from miR403, an active regulatory miRNA. The shRNA was 107 bp long and have overhangs for *Hin*d*III* and *XbaI* restriction sites (Table I). These oligos were annealed and cloned in pCDNA3.1 (+) vector (Invitrogen).

Gene amplification and construction of expression vector

Primers PVXIF and PVFIR (Table I) were designed to amplify ~891bp full length coat protein gene of *PVX* (CP-*PVX*) from *PVX* positive potato samples. Amplified product was cloned TA vector and sequenced. Deduced sequence was submitted to NCBI database. Further, for *in-vitro* knockdown assay, CP-*PVX* gene was directionally cloned in pCDNA3.1 (+) vector (Invitrogen) utilizing *Hin*d*III* and *XbaI* sites to obtain construct pVXC.

Cell culture and transfection

HepG2 cell line was maintained in DMEM media with 10% fetal bovine serum (Gibco) with penicillin and streptomycin antibiotic at 100 µg/ml. Cells were grown overnight and transfected at confluency with 3×10^5 cells per well in a 6 well culture plate. 50ng of pVXC DNA was transfect into the cell line and the different siRNAs at different concentration (10nM, 25nM, 50nM and 100nM) was subsequent co-transfected for 24 h. Transfection was mediated by Lipofectamine (Invitrogen) according to manufacture instruction. All experiments were done in triplicate.

Cells were grown overnight and transfected at confluency with 3×10^5 cells per 6-well format. The next day, co-transfections of pVXC and shRNA vector was done by using four different treatments. The first treatment (1:1) includes 100ng of pVXC and 100ng of shRNA, the second treatment (1:2) includes 100ng of pVXC and 200ng of shRNA, treatment 3 (1:3) includes 100ng of pVXC and 300ng of shRNA while the fourth treatment (2:1) includes 200ng of pVXC and 100ng of shRNA. Transfection was mediated by Lipofectamine (Invitrogen) according to manufacture instruction. All experiments were done in triplicate.

Expression analysis for CP-PVX *mRNA*

Total cellular RNA was isolated using TRIzol reagent (Invitrogen) 24 h/72 h post transfection and cDNA was prepared from 1µg of total RNA by using cDNA synthesis kit (Thermoscientific). Primers for Real Time PCR (RT-F and RT-R, Table I) were designed using primer 3 software (frodo.wi.mit.edu) to amplify a fragment of 120 bp. Real-time amplifications, using SYBR Green detection chemistry, were run in triplicate on thermocycler with pikoreal 3.1 software (Thermoscientific). Reactions were prepared in a total volume of 10 µl containing 1 µl cDNA, 0.5µl of each 10 pmoles primer, 5 µl of Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermoscientific) and 2.5 µl RNase/DNase-free sterile water (Qiagen). The cycling profile comprised of 35 cycles of denaturation at 94ºC for 30 sec, annealing at 56ºC for 30 sec and extension at 72ºC for 30 sec, after initial denaturation of 94ºC for 5 min.

The threshold cycle number (Cq) for transgene was normalized to the Cq for the GAPDH housekeeping gene simultaneously amplified with the samples. The knockdown efficiency of each siRNA/shRNA was calculated through Cq values compared to scrambled siRNA sample in a delta-delta Ct method.

Table I. Sequences of shRNA, siRNAs and primers with their identity used in the study. Amplified product size is also mentioned along with primer sequences. shRNA sequence contains siRNA 3 sense and antisense sequences (underlined) along with loop and 5′, 3′ flanking regions. These oligos also contain overhangs of restriction sites for cloning in expression vector.

Serial #	Name	Sequence (5'-3')	Product size
1	shRNA-sense	TCGAGAGAGTCGTATTACGTCTGAAGCTGAAATGAATTAC AACCCTTTTCATCAGAAAAACTGTTGTTGTTTGTTGA TTTCAGCTTCAGACGTTTAATCTGTCTTTC	
2	shRNA-Antisense	TCGAGAAAGACAGATTAAACGTCTGAAGCTGAAATGAAC AAACAACAACAGTTTTTCTGATGAAAAGGGTTGTAAT TGATTTCAGCTTCAGACGTAATACGACTCTC	
3	PVX1sense	AAGGAACTGGATGCTGACTAACCTGTCTC	
4	PVX1Antisense	AATTAGTCAGCATCCAGTTCCCCTGTCTC	
5	PVX2sense	AATCAGCAAGATTGAGGCTATCCTGTCTC	
6	PVX2Antisense	AAATAGCCTCAATCTTGCTGACCTGTCTC	
7	PVX3sense	AACGTCTGAAGCTGAAATGAACCTGTCTC	
8	PVX3Antisense	AATTCATTTCAGCTTCAGACGCCTGTCTC	
9	PVX-RTF	GGCAGCAGCAATTAAAGAGG	
10	PVX-RTR	GAAACCTTGTGCTTGCCAGT	120bp
11	PVXIF	GGACTGAACCTTGTGTCATCA	
12	PVXIR	ATGAAACTGGGGTAGGCGTC	891bp

Statistical analysis

One-way ANOVA and post Dunnet's test were applied on knockdown data obtained from real time PCR assays by employing Graph Pad Prism Software combined with Microsoft excel.

RESULTS

Oligos and construction vectors

A complete region of the *PVX* coat protein gene of 891bp was successfully amplified from *PVX* infected potato samples. The deduced sequences were submitted in NCBI Gene bank under accession # KC757709. Further, gene was successfully and directionally cloned under the influence of Cytomegalovirus promoter to make it expressed in mammalian cell line. Potential clones were confirmed through restriction digestion where the release of ~891 bp gene and ~5.4 kb vector confirmed its positivity (Fig. 1A). Similarly, shRNA oligo of ~107bp was cloned directionally in pCDNA3.1(+) and restriction digestion revealed two distinct fragments; one at ~ 5.4kb vector and 107 bp shRNA (Fig. 1B).

siRNAs selection based on their *in-silico* efficacy predict maximum knockdown values and favorable GC content of 42 - 47% along with antisense preference and no cross-silencing ability. The siRNA1 was nonspecific for CP-*PVX;* siRNA2 targets the CP gene at 404 bp with probable silencing score of 90; siRNA 3 target at 176 bp with probability score of 84 while siRNA3 target at 551 bp position in CP-*PVX* gene with 84 silencing score.

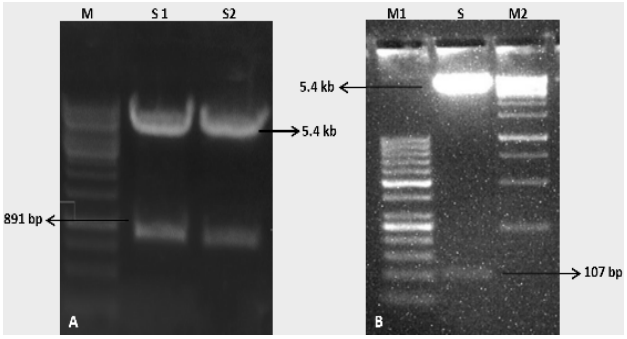

Fig. 1. Restriction digestion to confirm the expression constructs. (A) Restriction digestion of pVXC construct to reveal positivity of the clone. CP-PVX full length gene appears at 892 bp while pCDNA vector appears at 5.4 kb, (B) Restriction digestion of pSHX construct to reveal positivity of the clone. shRNA appears at 107 bp while pCDNA vector appears at 5.4 kb.

Inhibition of CP-PVX *gene expression in HepG2 cells*

Through semi-quantitative analysis, the expression level of CP-*PVX* gene in cultured cells was obtained at 1µg of DNA concentration while least was observed at 50

ng of DNA. Hence, for co-transfections, 50ng was used.

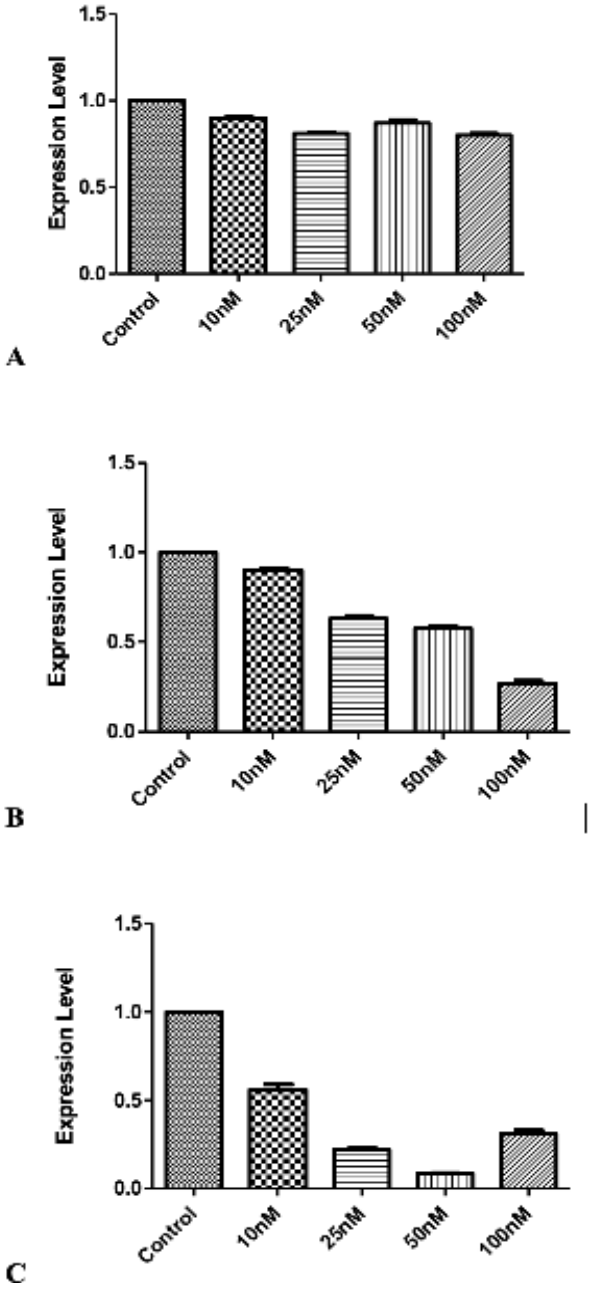

Fig. 2. Knockdown of CP-*PVX* mRNA by target specific siRNA in cultured mammalian cells 24 h post transfection. (A) CP-*PVX* mRNA knockdown caused by siRNA1 in HepG2 cells. (B) CP-*PVX* mRNA knockdown caused by siRNA2 in HepG2 cells and (C) CP-*PVX* mRNA knockdown caused by siRNA3 in HepG2 cells. One-Way ANOVA and post Dunnet's test were applied. The results showed that there was significant difference among control and different concentrations in standard value. ($P < 0.05$; $n = 3$).

The siRNA1 did not induce significant silencing in the CP-*PVX* gene 24 h post transfection (Fig. 2A). However, significant silencing effect was exhibited in siRNA2 and siRNA3 respectively as three concentrations. siRNA2 reduced the CP-*PVX* mRNA expression to 10% at 10 nM siRNA concentration; 42.4% at 25 nM; 36.39% at 50 nM and 73.04% at 100 nM siRNA concentration (Fig. 2B). Similarly, siRNA3 reduced the CP-*PVX* mRNA expression to 43.95% at 10 nM siRNA concentration; 77.80% at 25 nM; 91.91 % at 50 nM and 68.8 % at 100 nM siRNA concentration (Fig. 2C). The Cq values were normalized with GAPDH Cq values. Hence, the knockdown percentage of CP-*PVX* mRNA expression in HepG2 cells was least with siRNA1 when compared with siRNA2 and siRNA3 knockdown values. siRNA1 reduced the mRNA expression of CP-*PVX* by 1.25 folds while siRNA2 was able to reduce the mRNA expression by 3.7 folds. While in parallel, siRNA3 was found to reduce the mRNA expression of CP-*PVX* mRNA to a significant extent (12.5 fold). These values are in comparison to scrambled siRNA taken as control in these assays (Fig. 2).

For shRNA mediated knockdown assay, 72 h post transfection, variable knockdown was observed among the four treatments. It was found that 1:1 treatment reduced the CP-PVX mRNA expression to 31%, 1:2 decreased the mRNA expression to 62%, 1:3 exhibited maximum knockdown of 91% while 2:1 treatment reduced the mRNA expression to 16% only (Fig. 3).

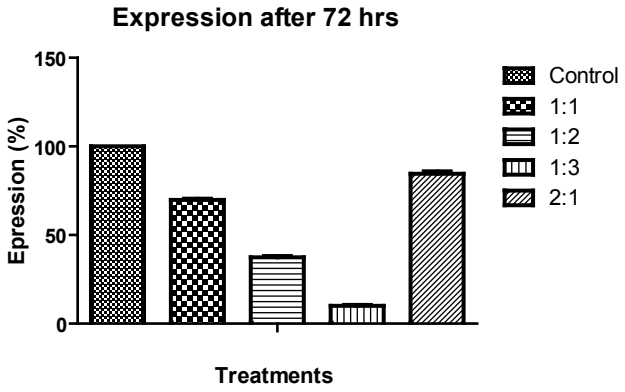

Fig. 3. Knockdown of CP-PVX by shRNA in HepG2 cells line. Four different traetments comprising of varied concentration of shRNA and target DNA were used to reveal mRNA suppression of coat protein gene of PVX. All samples were run in triplicate and normalized with GAPDH.

DISCUSSION

Over the past few decades, RNAi has emerged as a very powerful technique in functional genomics. The

ancient antiviral defense of the cell has been exploited to trigger specific gene inhibition (Mocellin and Provenzano, 2004). In this study, the coat protein gene of potato virus X was targeted due to its vital roles in virus accumulation, encapsulation and systemic spread (Kretschmer and Sczakiel, 2003; Korf *et al*., 2005; Echeverri and Perrimon, 2006). Several studies have employed Virus-derived coat protein gene as well as various virus derived genes to confer resistance against targeted virus in plants (Goregaoker *et al*., 2000; Kalantidis *et al*., 2002; Krulko *et al*., 2009). RNAi technology is a proven technique of generating virus resistant line in plant, and there is a great need to identified siRNA fragment that would produce the most effective and efficient result. RNA interference was successful used; to generate tobacco lines againts *PVY,* barley lines resistant to barley yellow dwarf virus *PAV* (*BYDV-PAV*), maize lines resistant to maize dwarf mosaic virus (Korf *et al*., 2005). We have identified best coat protein siRNA that can effectively be used for producing transgenic potatoes against potato virus X.

Transfection is a very powerful non-viral technology which is used to deliver foreign nucleic acid into eukaryotic cells to study relative expression and many other defined parameters. The generalized work plan for RNAi knockdown experiments involve the synthesis of siRNA for specific target, the delivery of siRNAs in cell line mediated through transfection reagent and the detection of percentage knockdown incurred by siRNAs. The same theme was applied in the current report where we initially optimized transfection conditions for transgene expression in mammalian cell line HepG2. Optimal transfection conditions are necessary to optimize for achieving relatively high transfection efficiency and for least cellular toxicity in cultured mammalian cell line (Missiou *et al*., 2004). As in cell culture systems, maintenance of lower cellular toxicity is very crucial parameter because transfection reagent mediated toxicity can mask the phenotypic expression of the target being tested. We have investigated different concentration of various siRNA to elucidate the least concentration for optimal knockdown efficacy to avoid chance of unwarranted toxicity.

Down-regulation of CP-*PVX* gene by small interfering RNAs (siRNA) resulted in the gene inhibition at post-transcriptional level. To trigger a strong RNAi activity against CP-*PVX* gene, screening of appropriate and effective RNAi target regions is mandatory. At present, there is a lack of clear understanding on the mechanisms that determine the gene-silencing efficiency of a given siRNA. Since not all cognate siRNAs that target mRNA are equally effective (Prins *et al*., 2008). Recent studies have shown that the gene silencing efficiency of siRNA is strongly dependent upon the local structure of mRNA at the targeted region (Scherer and Rossi, 2003). Out of three siRNAs, only siRNA3 that target CP gene at 551 bp position was capable of down-regulating the target mRNA expression to a maximum extent depicting that there is considerable variation in silencing efficiency among siRNAs. This variation may be, in part, attributed to the different accessibilities of siRNAs to the target mRNA (Novina *et al*., 2002; Mocellin and Provenzano, 2004; Pei and Tuschl, 2006; Parashar *et al*., 2013; Holen *et al*., 2002; Xu *et al*., 2003).

Maximum production of siRNA was detected in cells transfected with siRNA3 as compared to siRNA2 transfected cells. While siRNA1 treated sample exhibited minimum expression of particular siRNA indicating partial or minimum degradation of target mRNA. However, siRNA2 gave maximum knockdown of 73.04% at 100 nM concentration while for siRNA3, best knockdown of the target mRNA up to 91.91% was achieved at 50 nM concentration of siRNA. There was no change in the trend of knockdown expression at 24 h and 72 h of incubation time. siRNA3 reduced the expression of CP-*PVX* mRNA maximally, which resulted in the production of more target-specific siRNA corroborate similar findings reported by Jayan and Casey (2002) and Stewart *et al*. (2003). Our result suggest that the siRNA sequence is a vital factor that influence the potency and specificity of CP-*PVX* silencing.

CONCLUSION

We have developed a high-throughput, cell-based assay system for rapidly selecting and screening of siRNAs that effectively reduce mRNA. Identification of sensitive sites in the target RNAs regarding siRNA effectiveness is another significant achievement of this study for future resistance in potato against *PVX*.

Statement of conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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