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siRNA Silencing: mRNA Expression Knockdown of *Potato Leaf Roll Virus-Capsid Protein* Gene Co-Transfection with Mammalian (CHO-k) Cell Line *in-vivo*

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Abstract
Currently, the short interference RNA (siRNA) strategy has been used successfully to knock-down the mRNA expression of target virus genes. The target of this study was to provide a highly robust and concise methodology for screening of potent siRNA against of *Potato Leaf Roll Virus*(*PLRV*)-*Coat Protein*(*CP*) gene. In the present study, *PLRV-CP* gene amplicon of 346bp was used as a target mRNA for *in-vivo* knock-down. In total, four siRNAs were designed against the target gene and were subsequently screened in Chinese Hamster Ovary (CHO-k) cell line through transient transfection assays. The knock-down efficiency of *PLRV-CP* mRNA was analyzed by conventional reverse transcriptase-PCR and Real-time PCR assays. CHO-k cells were transfected with *PLRV-CP*gene construct and siRNAs simultaneously. Out of four, two siRNAs (siRNA1 & siRNA3) were found to be more effective or potent for knockdown of *PLRV-CP* mRNA expression in transfected CHO-k cells. *PLRV-CP*m RNA expression knock-down ability was found to be above 90%.Thesepotent siRNAs can be used for stable RNAi expression in plants that will become a powerful aid to probe gene function *in-vivo* and for gene therapy of diseases caused by viruses.

1. Introduction

Infections of plants with viruses are a serious impediment threat to agricultural productivity and a constant threat concern to global food security and hunger. *Potato Leaf Roll Virus* (*PLRV)* is of particular concern, because it is responsible for potato crop losses and found in all potato fields of the world. The diseased plants produce few tubers which smaller in size than normal plants, resulting in significant yield reduction. Due to the development of phloem necrosis, the tuber quality is also badly affected. In Pakistan potato virus is widely distributed in all potato growing areas with 15-65% incidence(1), which is again proved by a recent survey report study was conducted by Gul*, et al.,*(2011) and revealed that *PLRV* is widely distributed in all potato cultivation areas of Pakistan(2). For controlling this virus, it is strongly suggested to use the *PLRV* resistant potato cultivars. This virus encompasses one of the major threats in potato production and seed

potato certification. Major efforts have been provided in the development of effective strategies to reduce *PLRV* incidents, including vector control, and breeding for potato lines with *PLRV* resistance or reduced susceptibility. However, the control of *PLRV* has mainly relied on the release of certified *PLRV* free seed potato lots.

PLRV belongs to the family *Luteoviridae* and is a member of the genus *Polerovirus*. It has a non-polyadenylated, mono-partite and +ve sense RNA genome size is about 5.7 kb. A 5.9 kDa *PLRV* genome consists on one additionally acclimatized fiber RNA molecule that encrypts 6 Open Reading Frames (ORF).Three of those frames breadth assemblage is anchored abutting to the 3' end encodes RNA molecule (sub-genomic) and a covering of 23 kDa capsid protein (*CP)* or called supper molecule and a floematic movement of protein of 17kDa(3) and one 56 kDa super molecule anxious aural the vector relation(4).

The genomic assay of potatoes has lagged behind a lot of important crops. Advantageous cistron analysis in potatoes continues to be abiding adjustment and is usually bedfast by the complicated characteristics accompanying the potato adjustment like self-incompatibility, auto-tetraploidy and top state. Abounding methods are activated in addition to age-old breeding practices. Owning to these complexities, the authority of old strategies is quite poor. Therefore, an alternative access for any advancement of bread-and-butter type potato varieties is genetic transformation and the use of pathogen-derived transgenes to actualize adjoining potato aggressive viruses like *PLRV*. The RT-PCR offers an absolutely efficient and reliable procedure for detection of *PLRV*. So far, no reports are available about the availability of any approved potato variety in Pakistan with resistance against viruses(5). However, new methods are currently envisaged to architect virus resistance, accurate the house-keeping expression of an intron-containing self-complementary 'hairpin' RNA polymer (hpRNA), termed as PTGS system. RNA silencing implies the expression of a viral RNA/DNA sequence that does not need to be functional. It induces acceptance of virus sequences by the plant corpuscle and then affect its elimination. PTGS is triggered regionally and disentangle systemically throughout the plant via an adaptable silencing arresting that may cross grafting junction additionally(6).Admittedly not all accomplishment are apparent, however PTGS seems to be the foremost able. Arrangement with ability to knockout desired genes in transgenic plants. With the accumulation of techniques of *in vitro* amplification, biological analysis and genome-wide sequencing, the affairs of utilizing the accomplished communicable encourage adjustment for viral detection became possible. Inherent during this access is that the accident of abusage multiple genomic segments for creating specific and accepted detection probe. So as to actualize potato needs aggressive undertaking to combat a lot of severe infectious diseases like *PLRV*, a transgenic plant expressing working siRNA is needed. To assemble covering supermolecule (*CP*-protein) altercation transgenic plant, we will first appearance the siRNA adjoin the *PLRV-CP* genomic factor, employing a categorical criterion. Potent siRNAs are screened by co-transfecting siRNAsin affiliation with expression vector containing the *PLRV-CP* assembly into the CHO-k cell line. *PLRV-CP* gene abnormality altercation can be analysed by using either RT-PCR or Real-Time PCR or both.

In this study, siRNAs are used as guides to cleavage of viral homologues on infection by *PLRV*. In order to harness this technology, to protect valuable potato cultivars, robust and efficient transformation and regeneration systems are required. Targeted regions of the viral genome must be characterized for targeted gene silencing to allow for engineering of transgenes that can be used for RNA silencing of *PLRV* on infection.

The prime aim of the present study was to screening of effective or potent siRNAs against *PLRV*-coat protein (*CP*) gene *in vivo*. For taking the full advantage of gene silencing by siRNA, an appropriate screen system should be devised. The screening can be done by cloning of each siRNA and analyzing expression knockdown of the target gene in plant cell culture system, which is very laborious and time consuming(7) or by screening based on phenotype conferred by knock-down of the target gene. To overcome these laborious methodologies, we have optimized an efficient siRNAs screening technique by using transient expression in mammalian cell (CHO-k) line by this study.

2. Materials and Methods

2.1. Multiple Alignments

For designing the accurate and specific primers for amplification through RT-PCR, we aligned the subject *PLRV*-DNA sequence with the reported reference sequenced from National Centre of Biotechnology Information (NCBI). We selected the *PLRV-CP* gene sequence (Accession No. NC_001747.1, accessed on 2010/04/15) and aligned with 56 reported sequences. For further analysis and alignment, we took sequences with maximum homology (>96%) and query coverage (>97%) to our query sequence. We used Basic Local Alignment Search Tool (BLAST)for searching out the homologous sequence at Gen Bank nucleotide database**(8)**. The deduced, computationally, protein sequence was also aligned in the same method, first pairwise and then multiple alignments.

2.2. RT-PCR Amplification

For molecular characterization, we extracted total RNA from *PLRV* positive potato plants infected with local strain by using TRIzol reagent (Invitrogen). Complementary DNA (cDNA) was synthesized by using RNA as a template for RT-PCR amplification. We amplified *PLRV*-capsid protein gene. For this purpose, a fragment of *PLRV-CP* conserved gene sequence was retrieved for primer designing and multiple sequence alignment performed with Clustal-X virsion-2.0.

The conserved gene sequence was then used to design primers PLRV346-FP and PLRV346-RP (Table 1) by using Primer3 (Software, version 0.4.0)**(9)**. A primer sequence for virus was tested *in silico* using BLAST on GenBank to minimize the likelihood of non-specific amplification.

2.3. Cloning of *PLRV-CP* Gene in TA-Cloning Vector and Restriction Digestion with *EcoR1*

RT-PCR products were resolved on 1 % agarose gel and 346 bp fragments were cut for elution. Elution was done by using silica bead DNA gel extraction kit (Fermentas). The purified PCR product was ligated in TA cloning vector PCR®2.1(Invitrogen) in 1:1 ratio. *Escherichia coli* (strain DH5α) cells were made before ligation reaction and stored at-70ºC. Ligation mixtures were transformed in chemically competent *E. coli* cells by heat shock method according to manual protocol. Blue/white selection was used as a screening tool. Plasmid DNA was isolated from selected white colonies using GenJetMiniprep Plasmid Isolation Kit (Fermentas). Plasmid DNA was digested with *EcoR1* enzyme and fractionated on agarose gel.

2.4. Sequencing of *PLRV-CP* Gene

Automated DNA sequencing system (ABI 3700) from applied Bio-systems was used along with ABI PRISM genetic analyzer (Applied Biosystems, USA) using Big Dye™ terminator cycle sequencing Kit according to manufacturer's instructions. The *PLRV-CP* gene was sequenced using M13 primers (forward and reverse) respectively. The data was provided as fluorimetric scans, from which the sequence was assembled using the Sequence Navigator software. The nucleotide sequence was analysed using CHROMAS sequence analysis software version 2.0 and compared with *PLRV* sequences reported in GenBank database. Expert Protein Analysis System (ExPaSy) was applied to convert the DNA sequence into protein amino acid by prediction. In 6 frame results, we selected first 5'-3' frame protein sequence with lowest number of stop codons and position of stop codon in this sequence at 3' end. Protein sequence was aligned with other proteins sequence to find out the best and most suitable functional 3-D molecular structure of our *PLRV* coat protein. Structure with maximum homology was used as template for modelling.

2.5. pC*PLRV* Expression Plasmid Construction

For the effective and significant knockdown of *PLRV in-vivo*, *PLRV-CP* gene re-amplified by introducing *Bam*HI and *Xho*I sites in both reverse and forward primers (PLBamHI-FP and PLXhoI-RP, Table 1). Amplified fragment was gel-purified by using gel purification kit and inserted into the mammalian cell expression vector, pCDNA 3.1(+) (Invitrogen, USA), to obtain the plasmid construct pC *PLRV*. The cDNA clone of *PLRV-CP* was transformed by using *E. coli* competent cells and insertion of cloned fragment was confirmed by restriction digestion analysis.

Table 1. *Primer Sequences used in amplification, cloning, real-time PCR and internal control studies of PLRV- CP mRNA expression.*

Primer name	Sequence (5'-3')	PCR product size(bp)
PLRV346-FP	CAGGCGCCGAAGACGCAGAA	346
PLRV346-RP	TTTGGCGCCGCCCTTCGTAA	
PLBamHI-FP	GGATGGGATCCCAGGCGCCGAAGACGCAGAA	400
PLXhoI-RP	CCCTACTCGAGTTTGGCGCCGCCCTTCGTAA	
PL-RT-F	ACAAAGGACAGCCTCATTGG	101
PL-RT-R	TGGTAGGCCTTGAGTATTCCAT	
GAPDH-F	ACCACAGTCCATGCCATCAC	453
GAPDH-R	TCCACCACCCTGTTGCTGTA	

Table 2. *Sequences of siRNAs antisense and sense oligos designed against capsid protein(CP) gene of PLRV used in the study.*

siRNA oligos name	5'-3' Sequence
siRNA-PLRV-1 Antisense	AATTCGCCGCTCAAGAAGAACCCTGTCTC
siRNA-PLRV-1 Sense	AAGTTCTTCTTGAGCGGCGAACCTGTCTC
siRNA-PLRV-2 Antisense	AAGGATGGAATACTCAAGGCCCCTGTCTC
siRNA-PLRV-2 Sense	AAGGCCTTGAGTATTCCATCCCCTGTCTC
siRNA-PLRV-3 Antisense	AAGCATCTTACTTCAGTTCGTCCTGTCTC
siRNA-PLRV-3 Sense	AAACGAACTGAAGTAAGATGCCCTGTCTC
siRNA-PLRV-4 Antisense	AAGTATCATCCCTCCAGTCCTCCTGTCTC
siRNA-PLRV-4 Sense	AAAGGACTGGAGGGATGATACCCTGTCTC
Scrambled-antisense	AACCTGCATACGCGACTCGACCCTGTCTC
Scrambled-sense	AAGTCGAGTCGCGTATGCAGGCCTGTCTC

2.6. Small Interfering RNAs (siRNAs) Designing and Synthesis

siRNAs were designed against *PLRV- CP*RNA by using 346 bp fragment nucleotide sequence. To design siRNAs, software provided by Ambion, (USA) was used. For designing siRNAs, we selected a 50-100 nucleotide downstream region of the start codon with 35%-50 % G+C content. Stretches of 4 or more nucleotide repeats were avoided, and sequences that share homology with other related or unrelated genes of the same organism were also

avoided. The designed template oligonucleotides (DNA) for each siRNAs were custom synthesized from Fermentas (USA). All oligos were 29 bp in length with 8 nucleotides of the T7 promoter sequences added at the 3' end for final synthesis of duplex siRNAs (Table 2). Antisense and sense oligo templates were annealed to synthesize siRNAs by using siRNA construction kit (Ambion, USA) according to the user manual protocol.

2.7. siRNAs Stability Assay

Before transfection in mammalian cell, siRNAs stability was assaied. Annealed siRNAs were incubated at 37°C overnight and digested with RNase and DNase. For purification, siRNAs were washed with siRNA washing buffer according to instruction manual (Silencer siRNA constraction kit, Ambion, USA) and finally eluted in 100 µl of 75^{o}C Nuclease-free water. siRNAs concentration were quantified to read the absorbance at 260 nm in a spectrophotometer and finally converted into nmol concentration for transfection in cells. siRNAs also run in 5%agarose gel for checking the stability.

2.8. Mammalian Cell (CHO-k) Line Culture

The CHO-k cell line was kindly provided by Biopharmaceuticals Lab (CAMB, Lahore Pakistan). Cells were cultured and sub-cultured in DMEM F-12 media with 10 % FetalBovin Serum (FBS, Gibco), 100µg/mL of streptomycin and 100U/mL of penicillin.

2.9. Optimization of Transfection Condition

CHO-k cells were grown in 12-well plates. 5×10^{4}cells were seeded per well containing 1.0 mL of DMEM F-12 medium containing 10% FBS. At first, about 60%-70% confluence cells were transfected only with p*CPLRV* construct plasmid and concentration was used 50 ng, 100 ng, 250 ng and 500 ng. After optimization of p*CPLRV* concentration, again cells were transfected with p*CPLRV* constraction plasmid together with siRNAs. All transfections were performed using lipofectamine 2000 as transfection reagent (Invitrogen). For knock-down of*PLRV-CP*mRNA, 50 ng concentration of p*CPLRV* was used while the siRNA concentration was used 100 nmol/L. All transfection experiments were performed in triplicate.

2.10. Reverse Transcription PCR (RT-PCR) Analysis

RT-PCR analysis was performed to measure the mRNA expression of *PLRV-CP*during the knock-down study. Total cellular RNA was isolated from transfected CHO-k cells by using TRIzol reagent (Invitrogen). cDNA was synthesized using 1µg of total cellular RNA, 1µl OligodT primer and DEPC treated H_2O upto 12µl then incubation the reaction mixture at 70°C for 5 minutes followed by immediately chilling on ice. After addition of 4 µl of 5x reaction buffer, 2µl of 10mMdNTPs and 1µlRibolock^{TM} (Ribonuclease-inhibitor) (20u/µl) the reaction mixture was again incubated at 37°C for 5 minutes, and then added 1µl M-MuLV reverse transcriptase (200u/µl, Fermentas) to make total 20µl reaction volume. Finally incubated at 42^{0}C for 60 minutes and stopped the reaction by heating at 70^{o}C for 10 minutes and chilled on ice immediately. PCR was carried out with the corresponding PL *BamH*I-FP and PL*Xho*I-RP primers (Table 1). The PCR amplification profile was adjusted at 27 cycles and annealing temperature at 58°Cvia gradient PCR. After initial denaturation at 94ºC for 4 minutes in a thermal cycler starting with denaturation at 94ºC for 1 min, primer annealing at 58ºC for 1 min and extension at 72ºC for 1 min followed by final extension at 72ºC for 10 minutes. All samples were run in triplicate and normalized to GAPDH mRNA.

2.11. Real-Time PCR Analysis

For the real-time PCR analysis, Primer3 software provided by (http://frodo.wi.mit.edu/primer3/verified on 2011-10-29) was used to design specific PL-RT-F and PL-RT-R primers (Table 1), that could amplify afragmentsize of 101bp out of the 346bp conserved *PLRV- CP* gene portion. Applied Biosystem 7500 Real-time PCR was used to perform, using Maxima SYBR Green Master Mix (Fermentas). 1µg of cDNA was used in each reaction to study the knock-down efficiency of *PLRV-CP* mRNA mediated by siRNAs. The amplification profile was adjusted at 35 cycles: denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 35s after initial denaturation at 95°C for 10 min. The GAPDH was used as a control for normalization.

2.12. Evaluation of siRNAs Effect on Cell Viability

Effects of siRNAs on cell survibility were evaluated by using Trypan Blue according to the manufacturers' instructions. Cell was grown in 24 wells plate and siRNAs were added at about 70 -80% confluence level in different concentration (20 nm, 40 nm, 60 nm, 80 nm and 100 nm). After 24 hrs.of transfection, cells were harvested by using 100 µl of0.25% Trypsin EDTA solution in each well then incubation for 5 minutes at 37^{0}C with 5% CO_2 in a humidified atmosphere.

2.13. Statistical Analysis

The relative gene expression in real-time PCR and effect of siRNA on cell viability was analysed by calculating the standard deviation. The relative gene expression was done by using relative quantification (RQ) values in different samples and where each real-time PCR assay was performed in triplicate. For evaluation of siRNA effect on cell viability, three independent experiments were performed in triplicate on different days with control experiment (untreated) and data taken an average of nine data points coming from three experiments. For the purpose of this study, siRNAs were defined as toxic when the average (on the basis of standard deviations) cell viability showed below 75%.

3. Results

3.1. Multiple Sequences Alignments and RT-PCR Amplification of *PLV-CP* Gene

Multiple sequences alignments were done with the sequence of *PLRV-CP* (Accession number NC b001747.1) and all the reported sequences of viruses with "Clustal-W software", and conserved regions were identified to design primers for RT-PCR amplification. Naturally infected potato plants by aphid vector (*Myzuspersicae*) were used for total RNA isolation of *PLRV* virus. *PLRV*-RNA was used in reverse transcription experimentto form complementary DNA (cDNA) which was further used as template in PCR reaction for amplification of 346 bp fragment by using gene specific primers (PLRV-346-FP/ PLRV-346-RP). Gradient PCR was done to optimize annealing temperature at 55^{o}C. The PCR product was analyzed onagarose gel (1.5%) along with 50 bp DNA marker and the results are presented as figure 1.

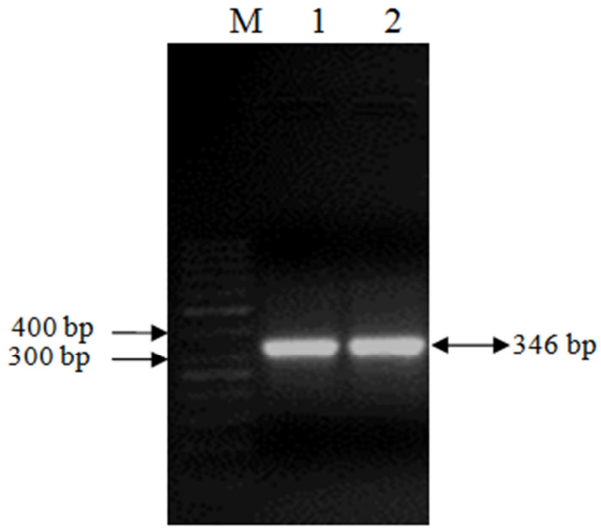

Figure 1. *Amplification of PLRV-CP gene through RT-PCR. M depicts 50 bp DNA Ladder while Lane 1-2 show PLRV-CP gene amplification.*

3.2. Cloning of *PLRV-CP* Gene into TA-Vector and Confirmation Through Restriction Digestion and Sequencing

Ligation of *PLRV-CP* gene was done into TA-cloning vector (PCR ® 2.1) at 1:1 vector to insert molar ratio and transferred through one shot chemical heat shock method inDH5α strain of *E. coli*. The screening of transformants was completed on LB agar plates having X-Gal (40 ug/ml) and kanamycin (50µg/ml) for selection of blue white colonies followed by isolation of recombinant plasmid. Different molecular techniques were applied to confirm the +ve clones. Cloned *PLRV-CP* gene was confirmed through restriction digestion analysis using restriction enzyme *EcoRI*and also via nucleotide and protein sequencing.

Isolated recombinant plasmids DNA (3µg) were digested with restriction enzyme *EcoRI*for the confirmation of cloned *PLRV-CP*gene. After two hours incubation at 37^{0}C the digestion reactions were checked on 1% agarose gel. *EcoRI* enzyme digested the plasmid and produced two bands; one was vector at~3.9kb whereas insert of the *PLRV-CP*gene was~346bp as shown in figure 2.

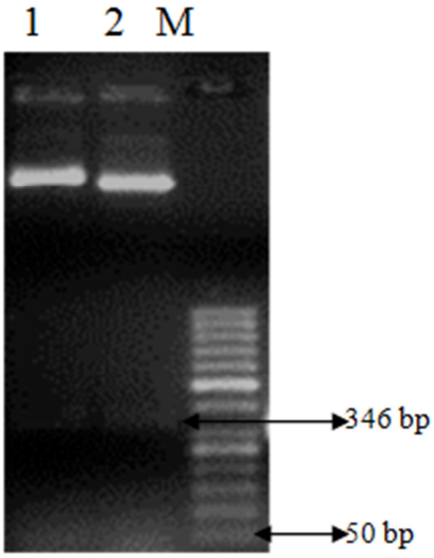

Figure 2. *Restriction digestion of the plasmid with EcoRI enzyme showing bands of vector (~3.9 kb) and insert (~346bp). M= 50bp DNA Ladder Lane 1-2: Plasmid digested with EcoRI.*

For the full-length sequencing, the *PLRV-CP* recombinant plasmid was used as a template with oligonucleotide (M-13) forward and reverse primers of this *PLRV-CP* cloned gene through automated DNA sequencing system (Applied Biosystems 3100 DNA Analyzer). Nucleotide sequence results of *PLRV-CP* gene was aligned with genes of other isolates of *PLRV* reported in GenBank database. This novel nucleotide sequence of *PLRV-CP* gene was submitted as a partial sequence to the NCBI database as Pakistani isolate of *PLRV*-coat protein gene. GenBank has provided the accession number (GenBank accession no. JN039286). The sequence of the accession # JN039286 is given as a figure 3.Predicted protein sequence of amplified fragment of *PLRV-coat protein (CP*) gene was revealed through ExPaSy Protein Translate software. The predicted protein sequence is given as figure 4.

CAGGCGCCGA AGACGCAGAA TAGGAGGCAA TTCGCCGCTC AAGAAGAACT GGAGTTCCCC
GAGGACGAGG CTCAAGCGAG ACATTCGTGT TTACAAAGGA CAGCCTCATT GGGCACTCCC
AAGGAAGTTT CACCTTCGGG CCGAGTCTAT CAGACTGTCC GGCATTCAAG GATGGAATAC
TCAAGGCCTA CCATGAGTAT AAGATCACAA GCATCTTACT TCAGTTCGTC AGCGAGGCCT
CTTCCACCTC CTCCGGTTCA TCGCTTATGA GTTGGACCCC CATTGCAAGT ATCATCCCTC
CAGTCCTACG TCAACAAGTT TCCAAATTAC GAAGGGGCGG CGCCA

Figure 3. *Nucleotide sequencing of PLRV- CP gene Pakistani isolate. (Accession No. JN039286).*

5'-3' Frame

QAPKTQNRRQFAAQEELEFPEDEAQARHSCLQRTASLGTPKEVSPSGRVYQTVRHSRMEYSRPTMSIRSQASYF

SSSARPLPPPPVHRLVGPPLQVSSLQSYVNKFPNYEGAAP

Figure 4. *Predicted protein sequence of 346 bp amplified fragment of PLRV-CP gene.*

3.3. Cloning of *PLRV*-CP Gene in Mammalian Cell Expression Vector pCDNA3.1

The amplified *PLRV-CP* gene was further sub-cloned in pCDNA3.1,a mammalian cell expression vector. The objective of cloning was to express gene temporarily in the mammalian (CHO) cell line in order to analyse its mRNA expression. For that purpose, the cloned *PLRV-CP* gene was amplified from TA-cloning construct plasmid by using amplification primers (PLRV-346 FP & PLRV-346 RP) modified by *BamHI and XhoI* restriction sites in them. Addition of restriction sites in primers makes this cloning directional. Gene was expressed under the control of Pcmv promoter.

3.4. pC *PLRV* Plasmid DNA Isolation and Confirmation of Cloning

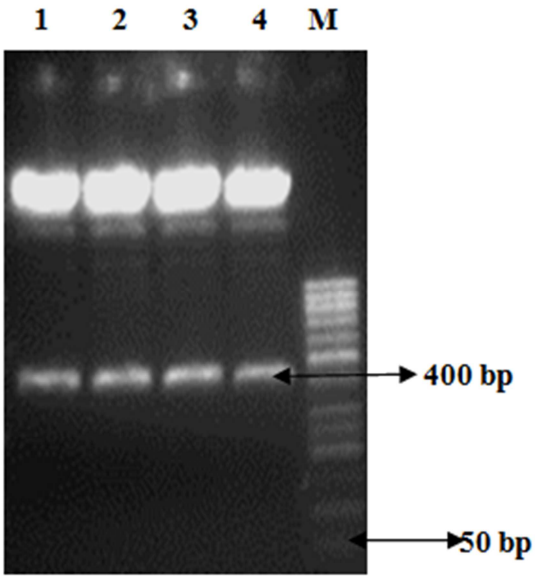

Figure 5. *pCPLRV constructs double digested by BamHI and XhoI enzymes. Depicted two bands; upper one vector pCDNA3.1 at 5.4 kb and lower one insert of PLRV- CP gene. Lane M: 50 bp DNA Ladder, Lane 1-4: pCPLRV constructs double digested, Lane M1: 1kb DNA ladder.*

The amplified *PLRV-CP* was ligated to empty vector pCDNA3.1 that restricted with *BamHI* and *XhoI* enzymes subsequently to transform in competent cells of E. *coli* (DH5α strain) and plated on kanamycin containing LB agar plates. Next day, collected transformed colonies and selected for inoculation to mini-prep culture. The resulting plasmid pCPLRV was isolated from mini-prep culture. The cloned gene into the pCPLRV construct was confirmed by double restriction digestion with *BamHI* and *XhoI* restriction enzymes and sequencing. Double digested products were resolved on 1.5% agarose gel which depicted two bands, one band at ~5.4 kb of vector pCDNA3.1, while the *PLRV-CP* gene was above 400 bp as clearly shown in the figure 5.

3.5. Transfection of pC *PLRV* Plasmid in Mammalian (CHO-k) Cell Line Culture

The pCPLRV construct plasmid containing insert of *PLRV-CP* amplified segment (346 bp) was transfected in mammalian cells line (CHO-k)with different concentrations of 50 ng, 100ng, 250ng and 500ng respectively and assay time allowed upto24 hours at control conditions. RNA expression of *PLRV-CP* gene in transfected CHO-k cell line was observed through RT-PCR analysis. The RT-PCR results showed low expressionas50ngconcentration of pCPLRV. But, it was noted that mRNA expression was increased proportionally with increased pCPLRV construct plasmid concentrations. RT-PCR depicted no expression, when the cells were transfected with empty vector pCDNA3.1 (+) and in control (untreated) cell line. These results are clearly shown in figure 6. For this transfection study, lipofectamine 2000 was used as transfection reagent and optimized the concentration.6 µl of lipofectamine per 12-well culture plate was given the best result with 50ng pCPLRV construct plasmid concentrations. It was considered that in CHO-k cell line with GAPDH as internal control, 50ngpCPLRV plasmid concentrations along with 6µl lipofectamine per 12-well plates was optimum expression. In case of GAPDH, it was found that mRNA expression was all most same in all cases including transfected and non-transfected with free pCDNA3.1 vector as seen from the figure 6 of GAPDH RT-PCR results.

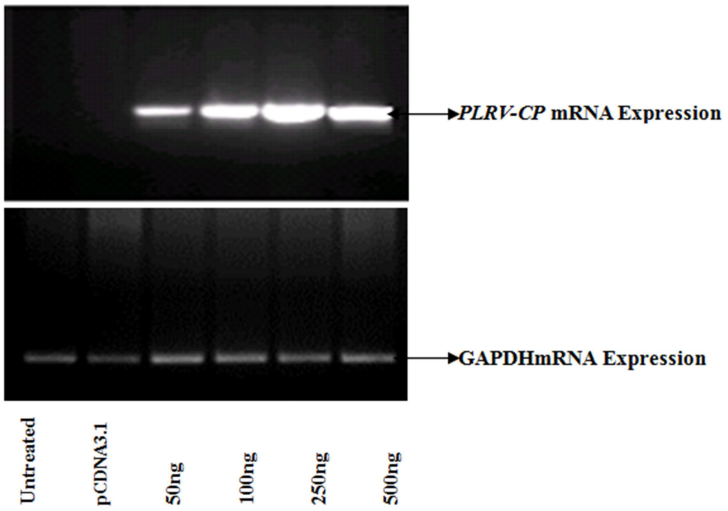

Figure 6. *mRNA expression of RT-PCR analysis for optimization of PLRV-CP transfection conditions in CHO-k mammalian cell line. Lane-1: Untreated control (Non-transfected), Lane-2: Cell transfected with empty vector pCDNA3.1, Lane 3-6: Transfected with various concentrations (50, 100, 250 and 500ng respectively) of pCPLRV construct plasmid. GAPDH used to normalize the samples.*

3.6. Effects siRNAs on *PLRV-CP* mRNA Expression *in-vivo*

In the *in-vivo* study of mRNA expression knock-down of *PLRV-CP* gene, siRNA (100nmol/L conc.) was co-transfected along with previously optimized transfection conditions. RT-PCR results showed that all four siRNAs down regulated mRNA expression, but siRNA1 and siRNA3 substantially decreased *PLRV-CP* mRNA level. It was observed that the expression level was very low, when the cells co-transfected with siRNA1 and siRNA3 as matched to distorted siRNA (transfected with 50ng of pCPLRV) and positive control (transfected only 50 ng of pCPLRV but no co-transfection with siRNA). There was no expression in negative control, these RT-PCR results depicted in figure 7. The real-time PCR studies also confirmed the RT-PCR results, therefore, it was observed that in case of siRNA1 and siRNA3, *PLRV-CP* mRNA expression knock-down was above 90% and other two siRNAs knock-down values were about 75-80% as shown in graphical presentation in figure 8.For the expression of GAPDH mRNA, the siRNA transfected cells were also tested by using cDNA derived from the total RNA extracted from siRNA transfected cells. It was observed that the expression level was almost same in both types of cells, transfected with pCPLRV plasmid construct and non-transfected.

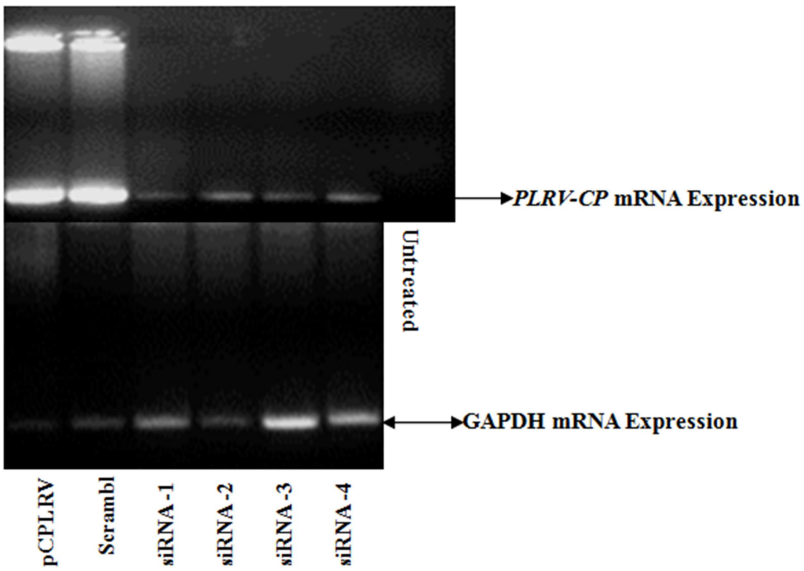

Figure 7. *In-vitro study of mRNA expression knockdown of PLRV-CP gene by selected siRNAs in mammalian cells line (CHO-k). The CHO-k cell line was cultured in 12-well plates and co-transfected with 50ng pCPLRV plasmid constructs and 100nmol/L of siRNAs. PLRV-CP mRNA expression; Effect of siRNAs on mRNA expression in CHO-k cell line, analyzed by RT-PCR. GAPDH mRNA expression level as internal control.*

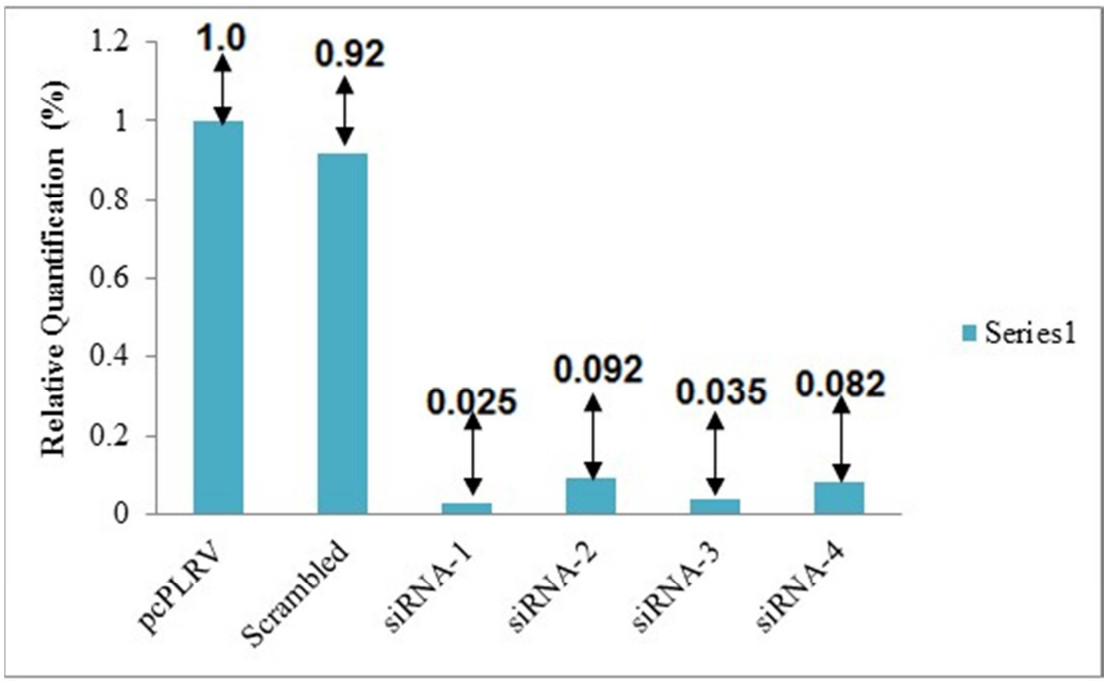

Figure 8. *Real-time PCR analysis, effect of siRNAs on PLRV-CP mRNA expression knockdown in CHO cell line. Results were taken from three independent experiments in triplicate. GAPDH was used as internal control.*

3.7. Toxicity Experiment of siRNAs at 100 nmol/L Concentration

In this study, toxicity experiments of siRNAs were also conducted to estimate the cells viability percentage upto 100 nmol/L siRNAs concentration. For this purpose, siRNAs were added to the cells at about 70-80% confluence level with five different concentrations (20nm, 40nm, 60nm, 80nm and 100nm).After 24 hours of transfection, cells were harvested by using Trypsin EDTA and counted through tryptone blue experiment. The experimental results showed not much significant difference among the treatments and also with control experiment (untreated), as indicated in the graphical presentation in figure-9. It means that the siRNAs were not toxic to the cells at 100nmol/L concentration. Three independent experiments were performed in triplicate with control experiment (untreated).

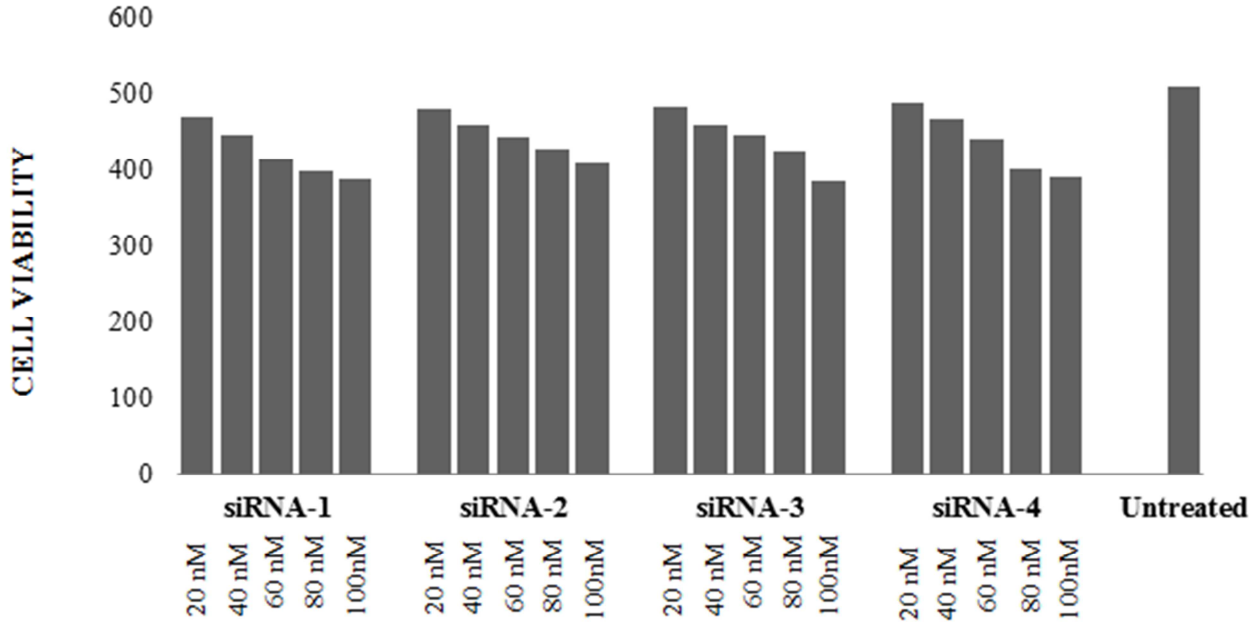

Figure 9. *Estimation of siRNAs toxicity on cell viability percentages through trypan blue experiment. Series 1-5 represented the low to high siRNAs concentration (20nm, 40nm, 60nm, 80nm, 100nm).The selected siRNA concentration (100nm)showed not too much significant different among the treatments and also not with control treatment (untreated)after 24 hours of transfection.*

4. Discussion

Among the most important food crops, the potato is always present, not just in Pakistan, but all around the world. There are number of pathogens effecting potato yield like viruses, fungi, bacteria, phytoplasma, physiological disorder etc. Potatoes are infected by more than 50 viruses and phytoplasma diseases world wide (10), and *PLRV, PVY,* and *PVX* are the severely affecting potato viruses that reduce potato yield by10-90%(11). To date, farmers are using various insect controlling pesticides to minimize the chances of virus attack. Butit is not possible to solely control *PLRV* by direct application of chemical insecticides as it increases the cost-benefit ratio for farmers and producers. For the control of viral attack, cultivation of virus resistant potato variety or line is the only sustainable and environment friendly. The development of transgenic potato crop with virus-resistance is an acute charge of the time and of abundant accent for a top potato crop in the country.

Saiki *et al.,*(1985) described that successful PCR reaction is because of the choice, quality & accuracy of various factors, liketemplate DNA/cDNA (RT-PCR), primers, dNTPs, concentration of magnesium ion, choice of polymerase enzyme and primer's annealing temperature(12). As a first step of this study, we develop RT-PCR mediated commercial scale screening protocol of *PLRV*.As RT-PCR gives higher sensitivity with high speed diagnosis and reduced sample size, therefore, it was found good alternative to other diagnostic methods like ELISA, etc. PCR assays for *PLRV* detection were made quite easy and possible by the database of nucleotide sequences for many plant pathogens like viroids, viruses, etc. Similarly, a number of scientists have reported the usefulness of RT-PCR for the detection of many plant viruses e.g. apple scar skin, grape vine virus A, pome fruit virus, and potato virus A from dormant tubers(13, 14, 15, 16). It has been reported that Multiplex RT-PCR was also used detection of five potato viruses, i.e. *PLRV, PSTV, PVX, PVS* and *PVA* simultaneously(17).There are other reports regarding efficient detection *PLRV* in dormant tubers by real time PCR, but it costs are very high(18, 19).The RT-PCR based detection procedure is seems to be cost effective and applicable. Schoen et *al.,* (1996) also suggested that, as a reliable and effective test, the RT-PCR method should be used for routine diagnosis of potato viruses because it showed higher sensitivity(20). So we concluded that, for epidemiological studies of *PLRV*, this molecular technique is a reliable method for potato seed (tuber) certification programs and quick diagnoses of *PLRV* from potato. One of the objectives of this study was the characterization of local *PLRV* isolate through cloning and sequencing of amplified *CP*-gene fragment. For this purpose amplified *PLRV-CP* gene was cloned in molecular vector. The cloned *PLRV-CP* gene was confirmed through restriction digestion and its nucleotide sequencing. The sequence of local *PLRV* isolate showed 94-97% homology with already reported *PLRV* nucleotide sequences in GenBank databases, which also showed its relative conserveness with *PLRV* genome as documented in previous reports(21). Maximum homology was noted with Canadian isolate as well as European isolates, and distant relationship was seen when compared with Australian isolate.

Although severely damaged by this virus, the potato remains a very economically important crop. The main target of the present study was to use siRNA for developing a genetically engineered potato cultivar that would be strongly resistant to *PLRV* infection. To achieve this goal, we have introduced a new method of transient expression in CHO-k cell line *in-vivo* for screening the most effective siRNAs from a larger pool as efficiency of siRNAs may vary a lot. *PLRV* belongs to the *Luteoviridae* family and is a member of the *Polerovirus* genus. The unique characteristic of *Polerovirus* is that aphids transmit this virus in a circulative way. So it is non-mechanical in transmission and non-propagative in manner. Viral infection is only found in phloem level (22). Major protein (CP) and minor protein (P5) is encoded by *PLRV* as two coat proteins. Another CP version with extended residues is produced through rare translational 'read through' of the *CP* gene (23). In this study, we selected major protein/coat protein gene of *PLRV*, to confer resistance against *PLRV*. The coat protein (*CP*) gene fragment can confer resistance/immunity in transgenic plants, because these can completely suppress virus by dsRNA through pathway of RNA silencing (24, 25, 26, 27). To design siRNAs, in this study, we selected 346bp*PLRV-CP* gene fragments as a template. In the multiple sequence alignments study, this sequence found strongly conserved among the *PLRV-CP* genes with NCBI reported *PLRV* isolated sequences comparison. This *PLRV-CP* gene fragment was considered as complete *CP* gene template. This fragment was used to design siRNAs. It has a 94%-97% resemblance with all reported sequences in GenBank. Thus through the screening studies of this particular siRNA we obtained evidence of a resistance that would be equally effective against all of the *PLRV* isolates. These siRNAs were screened using transient expression studies in CHO-k cell line. The two siRNAs (siRNA1 and siRNA3) were found to down regulate the *PLRV-CP* mRNA expression above ~90%. These results were found through RT-PCR analysis. In this research, we employed the mammalian cell line (CHO-k), because they are a stable and robust cell line giving expression within 24 hours. Plant cells were not used, as they cannot be processed in a panel because they need to be individually cloned for each siRNA. Therefore, expression analysis becomes a tedious and difficult procedure for siRNA transformation in plant cells. Different authors have confirmed that the siRNA silencing mechanism is almost the same against viral genes in both plant and mammalian cells (28, 29, 30). Due to this, we selected the mammalian cell line (CHO-k) for siRNA testing and selection rather than plant cells. Sequence-specific mRNA control is the basic strategy of RNA silencing. In plants it is also referred to as RNA-mediated resistance or Post-Transcriptional Gene Silencing

(PTGS). In animals it is referred to as RNA interference (RNAi) and in fungi it is referred to as silencing (31). Moreover, genetic and biochemical analyses have proved that the main procedure of RNA silencing pathways is the same in higher organisms (32,33,34, 35, 36). Furthermore, specificity of siRNA knock-down to pCPLRV construct was observed i.e. against *PLRV-CP* transgene; there was no mRNA (viral) in cells transfected with pCDNA 3.1 (+) empty vector or in untreated CHO-k cells when the experiment's outcomes were checked by RT-PCR and again confirmed by Real-time PCR analysis. A clear understanding of how the *CP* gene was specifically knocked-down can be obtained through a proper concept of homology. Our results are similar with the RNA silencing mechanisms already found by Miyagishi and Taira 2002; Brummel Kamp *et al.,*2002 and Elbashir *et al.,* 2001(37, 38, 39).

5. Conclusions

According to the above discussion, we can conclude that development of a method for screening the most potent siRNA from the four siRNAs designed to influence biological functions, which can significantly decrease activity of the target genes. This new technology enables us to establish i) a system for expression of specific gene (s)in cell culture condition and ii) a potent siRNA screening through the bulk panel method for robust and precise results. Use of siRNA for stable RNAi expression in plants is a promising strategy for investigation (*in-vivo*) of gene functions and cures for viral diseases.

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