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Original Research Article

A computational approach to execute siRNA generating hotspots targeting dual DNA and RNA viral infections in potato

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Abstract

Among biotic stresses afflicting potato plants, viruses are the most damaging and are responsible for large economic losses worldwide. Co-infections with multiple viruses are common in potato, with an enhanced disease impact being observed in affected plants. RNA interference (RNAi) provides an applied methodology to selectively reduce the expression of targeted genes through the expression of sequence-specific short interfering RNAs (siRNAs). This silencing mechanism can be implemented to induce resistance against multiple viruses in transgenic plants through the endogenous delivery of siRNA cassettes. The current study was aimed to identify the efficient siRNA execution sites in dominating viral genomes to simultaneously target both DNA and RNA viruses in potato. To achieve this objective, we followed a computational approach to identify the viral silencing targets by comparative pairwise sequence analysis of different isolates of *Potato leafroll virus* (PLRV; + single-stranded (ss) RNA virus) and *Tomato leaf curl New Delhi virus* (ToLCNDV; ssDNA virus). The identified consensus sequences [300bp of PLRV-coat protein (CP); 180bp of ToLCNDV-precoat protein (AV2)] were further used as template sequences to predict the likely siRNAs execution sites and to characterize their putative thermodynamic attributes. The identified template sequences were computationally tested for triggering a siRNA-mediated targeting of viral genomes and proved to be highly efficient and site-specific. This methodology could be applied for engineering an RNAi-mediated virus resistance in transgenic plants with commercial applications.

Keywords: Potato, RNAi, Resistance, siRNA, Virus

Introduction

Potato (*Solanum tuberosum* L.) is a solanaceous tuber that is the world's leading non-grain food crop. The high food yield per unit area of land and time has enabled potato to mitigate the threats of global food security. Viral pathogens are the most important biotic factors that severely limit crop productivity. Potato is susceptible to around 40 different viral species, most of which are RNA viruses such as *Potato leafroll virus* (PLRV; genus *Polerovirus*), which is widespread in the potato-growing areas of the world (Hameed et al., 2014). Yield losses due to PLRV infections range from 20-90% due to severe leaf curling and tuber necrosis and results in internal damage of tuber tissues (Douglas and Pavek, 1972; Halim, 1999). PLRV is transmitted in a persistent manner by aphids, in particular, the green peach aphid (*Myzus persicae*),

which accelerates the viral dispersal from the field to field (Ragsdale et al., 2001). Over the last two decades, begomoviruses (family *Geminiviridae*) have emerged as the most geographically diverse plant viruses. They are critically destructive to numerous crop plants, including potato, cotton (*Gossypium hirsutum*) (Zaidi et al., 2016), tomato (*Solanum lycopersicum*) (Kanakala et al., 2013), and pepper (*Capsicum annuum*) (Singh et al., 2016). *Tomato leaf curl New Delhi virus* (ToLCNDV) is a bipartite begomovirus that infects potatoes in Indian sub-continent and severely limits crop productivity (Hameed et al., 2017; Usharani et al., 2004; Padidam et al., 1995).

Viral co-infections are epidemiologically increasing in nature and appeared remarkably more damaging compared to single-virus infections in diverse host species (Lamichhane and Venturi, 2015; Tollenaere et al., 2016). Several reports of co-infections among different viral species such as *Beet yellows virus* (BYV) and *Beet mosaic virus* (BtMV) (Wintermantel 2005), *Tomato chlorosis virus* (ToCV) and *Tomato spotted wilt virus* (TSWV) (García-Cano et al., 2006), and *Begomovirus* and *Sweet potato chlorotic stunt virus* (SPCSV) (Cuellar et al., 2015) have emerged as severe disease complexes that lead to breakdown of host resistance. Mixed infections of RNA viruses in potato are well-documented to produce synergistic interactions among different viral species that result in profound enhancement of disease etiology (Hameed et al., 2014; Syller, 2014). The transmission of viral diseases is generally dependent on insect vector activity, and most of the potato-infecting viruses depend on aphid species for their dispersal (Dáder et al., 2017). Crop rotation has allowed viruses to survive throughout the year on sequential hosts (West, 2014). Potato is short duration crop, being cultivated in rotation with other crops such as cotton, wheat, and rice. This leads to the potential risk of co-infections by RNA and DNA viruses, which may result in unpredictable pathological outcomes and severe crop failures.

During co-infections, viruses invade their host cells and express proteins that are responsible for viral replication, movement, pathogenicity, and encapsidation (Fig. 1A). In response to the viral attack, host cells activate defense responses which lead to an arms race between viral pathogenicity and host immunity. The synergistic interactions among co-infecting viruses often overcome host immune responses and emerge into new disease complexes (Lamichhane and Venturi, 2015).

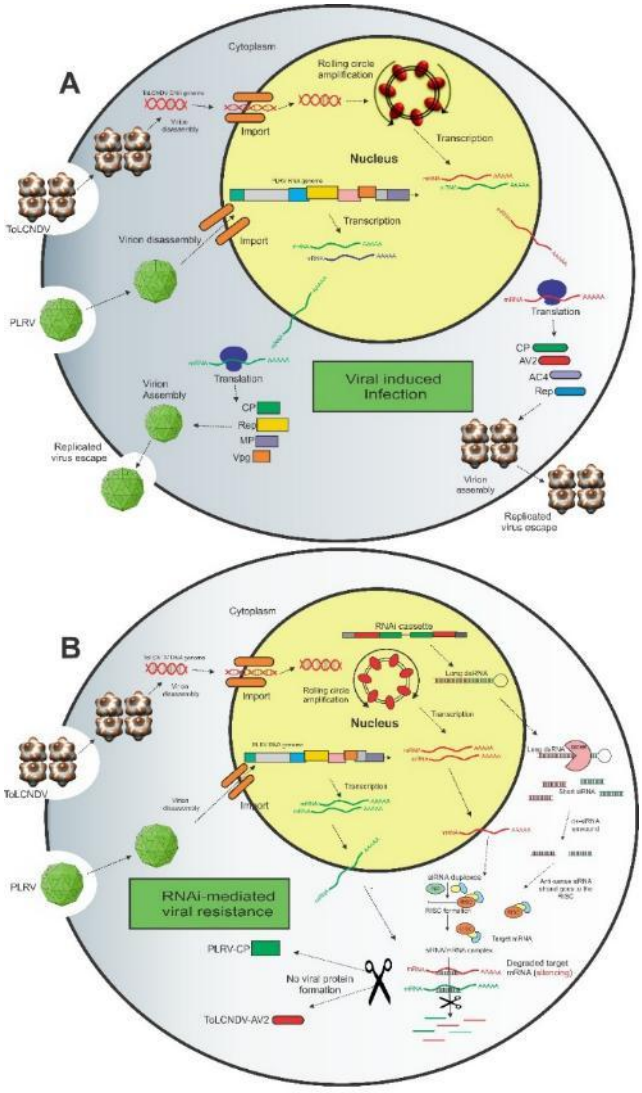

Figure - 1: Schematic representation of viral co-infections in non-transgenic and transgenic plant cells based on proposed RNAi-mediated methodology.

(**A)** In natural condition, multiple viruses (e.g. ToLCNDV and PLRV) invade plant cells and induce a successful viral infection after suppressing host immune responses. (**B)** In transgenic cells, an RNAi-mediated cassette triggers a homology based silencing mechanism where siRNAs will be generated. These putative siRNAs would target the sequence-specific viral mRNA transcripts (ToLCNDV-AV2 and PLRV-CP) leading to mRNA degradation or might initiate the post transcriptional gene silencing (PTGS) resulting in successful RNAi-mediated viral resistance.

RNA silencing offers a promising strategy to induce resistance against multiple viruses in transgenic plants (Saurabh et al., 2014). Through RNA interference

(RNAi) or post-transcriptional gene silencing (PTGS), endogenous pathways provide an effective and adaptable platform for targeted gene "knock-down" through homology-based degradation of cognate mRNA transcripts that is primarily triggered by short double-stranded (ds) RNAs duplexes (Agrawal et al., 2003; Mansoor et al., 2006). The degradation of intruding viral genome (RNA or DNA) in host cells can be achieved by expression of RNAi-mediated cassettes that will generate short interfering RNAs (siRNAs) (Fig. 1B). These siRNAs target the viral domains based on sequence homology and initiate an RNA-induced silencing complex (RISC) that leads to successful virus resistance (Fig. 1B).

Reports of RNAi-mediated resistance against diverse species of plant viruses show varying levels of success (Chung et al., 2013; Hameed et al., 2017; Mansoor et al., 2006; Zhang et al., 2011). The emerging challenges of plant disease complexes have emphasized the importance of generating broad-spectrum resistance against co-infecting viruses in a number of crop plants (Lamichhane and Venturi, 2015). In this context, RNAi technology offers a strategy to knockdown co-infecting viruses at the molecular level. Here, we have adopted an *in silico* strategy to initiate a siRNA silencing mechanism for inducing simultaneous resistance against PLRV and ToLCNDV infections in potato. For this purpose, we computationally identified the most appropriate siRNA generation hotspots in viral genomes and performed computational modeling to predict the potential *siRNA:mRNA* target duplexes. Furthermore, we have discussed the potential applications of this preliminary data to engineer a dual viral resistance in transgenic plants.

Materials and Methods

Sequence retrieval and multiple sequence alignment: Full-length genomic sequences of 10 different isolates of PLRV and 10 different isolates of ToLCNDV were retrieved from the GenBank database (NCBI, www.ncbi.nlm.nih.gov) (Table - 1). All retrieved sequences were aligned pairwise using CLC Main Workbench (version 7.8.1, www.qiagenbioinformatics.com) to identify the most conserved regions. The multiple sequence alignment (MSA) results led to the selection of the coat protein (CP) gene for PLRV and the precoat protein (AV2) gene for ToLCNDV. Furthermore, a subset of highly conserved sequences of PLRV-CP and ToLCNDV-AV2 were identified (Fig. 2). Based on the MSA results of selected PLRV-CP and ToLCNDV-AV2, consensus sequences were generated based on the most conserved nucleotides. These consensus sequences were further used as template sequences for siRNA computational modeling.

***In silico* retrieval of potential siRNAs:** To identify the likely siRNA execution sites in the viral consensus sequences, we employed different online siRNAs designing platforms, depending on the selection criteria for efficient siRNA duplexes (Birmingham et al., 2007). Subsequently, we used three siRNA designing tools: (i) DSIR, (Designer of Small Interfering RNA, www.biodev.extra.cea.fr/DSIR/DSIR.php) (ii) Invitrogen Block-iT RNAi Designer, (www.rnaidesigner.thermofisher.com/rnaiexpress); and (iii) pssRNAit: plant short small RNA interfering tool, (http://plantgrn.noble.org/pssRNAit/). The consensus sequences of PLRV and ToLCNDV were used as a template for siRNA retrieval on different platforms (Vert et al., 2006). Moreover, we utilized the filtering options of these programs for predicting the off-site targeting in the potato (*Solanum tuberosum* L.) genome.

Prediction of hybridization plot of *siRNA:mRNA* target duplexes *:* For calculating the secondary structures of targeted viral mRNAs, an online bioinformatics tool (RNAfold) was used. The nucleotide sequences of guide strands of the template sequence of PLRV and ToLCNDV were pasted in RNAfold. A graphical output of minimum free energy (MFE) structure was plotted to estimate the optimal secondary structure of transcribed mRNA of targeted nucleotide sequences. To predict the interactions of *siRNA*:*mRNA* target duplexes, an online tool (RNAup) was used using siRNA antisense strand and mRNA sense strands as template queries. RNAfold and RNAup programs were used online from following web server "The ViennaRNA Web Services" (www.rna.tbi.univie.ac.at).

Results

Analysis of viral conserved genomes

The pairwise MSA results of viral sequences (Table - 1) resulted in the identification of CP of PLRV and AV2 of ToLCNDV as the most conserved region in respective viruses (Figure - 2). Furthermore, a subset

having more than 99.9% sequence identity within the selected PLRV-CP (Fig. 2A) and ToLCNDV-AV2 (Fig. 2B) was identified. The selected highly-conserved consensus sequences (PLRV-CP, 300bp; ToLCNDV-AV2, 180bp) were further used as template sequences (Table - 2) for siRNA execution. To estimate the phylogenetic relations among the selected viral strains, a neighbour joining (NJ) phylogenetic tree was constructed following the MSA results using ClustalW in MEGA6 software (Supplementary Figure - 1). The phylogenetic dendrogram revealed the close relationships among the selected viral strains confirming their common origin.

Execution of putative siRNAs avoiding off-targets

The generated consensus sequences were utilized as input template sequences in different online RNA design servers to predict the functional siRNAs from these regions. For comparison, we utilized three online platforms (DSIR, Block-iT RNA Design, pssRNAit) to design potential siRNAs (data not shown). Through a comparative siRNA designing search, we identified pssRNAit as an effective tool for executing siRNAs on the basis of the numerous available features in this program. This program has a built-in feature of selective filtering for designed siRNAs to access any off-site hybridization with available host genomes by using a Nucleotide BLAST (BLASTn) analysis. The off-target accessibility of putative siRNAs is the most significant feature of pssRNAit which makes it a good choice over other programs (i.e. DSIR and Block-iT RNA Design). On the basis of generated data, we identified siRNAs having predicted higher efficiency (Table - 3) and were compared with siRNAs produced by other two programs (i.e. DSIR and Block-iT RNA Design) (Data not shown).

Thermodynamic attributes of designed siRNAs

On the basis of efficiency score ranging from 0-10 (higher value means high targeting efficiency), a subset of 5 putative siRNAs out of 19 generated siRNA for PLRV-CP and 4 putative siRNAs out of 5 generated siRNAs for ToLCNDV-AV2 template sequences were selected (Table - 3). The representative siRNAs with their nucleotide sequence and other thermodynamic characteristics are summarized in Table - 3. The underscored parameters such as GC ratio, off-target accessibility, unpaired energy (UPE) value, efficiency value, and RISC binding scores were considered for final selection of siRNAs. In addition, a higher A/U content at the 5′ end of the antisense strand and a higher G/C content at the 5′ end of the sense strand was considered important for siRNAs selection. Stretches of >4 T/A nucleotides at the 5′ end of the antisense strand of putative siRNAs were avoided to minimize the probability of transcription termination for RNA pol III. The GC ratio of selected siRNAs lies within a moderate range of 32-65%, as quantified by a GC content calculator (Table - 3). The siRNA efficiency score is another important feature that determines the functionality of siRNAs in target hybridization and off-target accessibility. In the present study, an efficiency score having a value greater than 6 was selected as a cut-off value for siRNAs selection (Table - 3). Another important feature of the RISC binding score prediction represents the probability of siRNAs incorporating in RISC silencing complexes. Here the RISC score of each putative siRNA was calculated by the pssRNAit program and is summarized in Table - 3. The UPE value of siRNAs, which scores the free energy required for siRNAs to open up their secondary structure in order to bind to the targeted mRNA sequence, is presented in Table - 3. The UPE value of siRNAs lies between 10-16 kcal/mol (Table - 3), which is a fairly low range for siRNAs to hybridize effectively with targeted viral mRNA transcripts.

Prediction of targeted mRNA secondary structure and hybridization plot analysis

The computational prediction of secondary structure of RNA duplexes provides a graphical representation of RNA-RNA interactions that might be involved in RISC complexes. To predict the targeted mRNA secondary structure, RNAfold was utilized having a minimized folding free energy value at 37°C. The siRNA target sites (red) show the predicted hybridization of *siRNA*:*mRNA* target duplexes which has an important role in siRNA efficiency (Fig. 3A for PLRV mRNA secondary structure; Fig. 3B for ToLCNDV mRNA secondary structure). To assess the probability of siRNA interaction with targeted mRNA sites, a hybridization plot was generated against the specific energy (ΔGi) of interaction (Fig 3 C & D). The plot demonstrates that the specific energy (ΔGi) required for antisense siRNA self-interaction is much lower than the energy required for mRNA self-interactions, which indirectly reflects the probability of *siRNA*:*mRNA* target duplex formation after opening the secondary structure around the targeted mRNA sites.

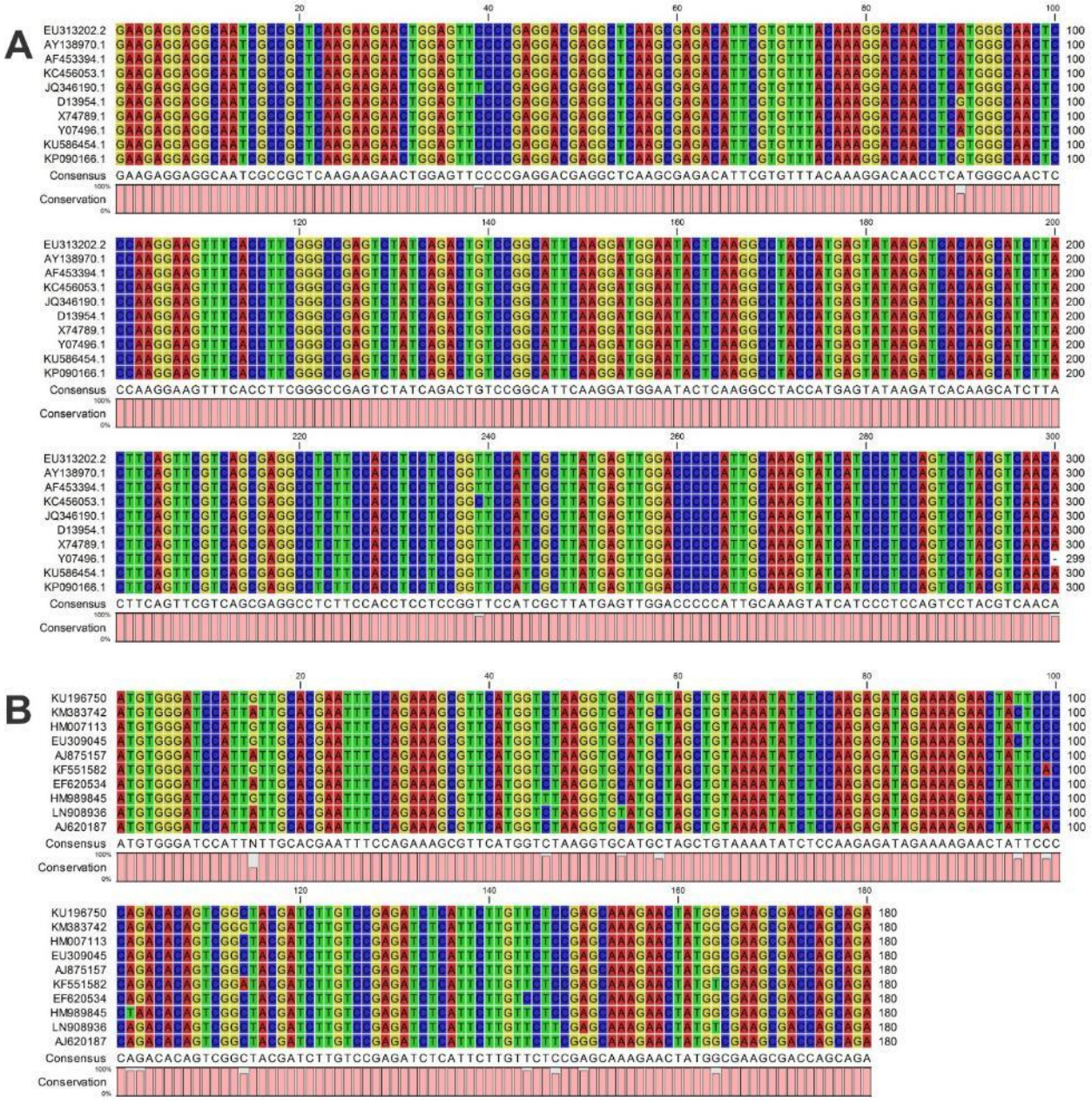

Figure - 2: Multiple sequence alignment result of
(A) Coat protein (CP) region of PLRV isolates
(B) Multiple sequence alignment result of AV2 gene region of ToLCNDV isolates.

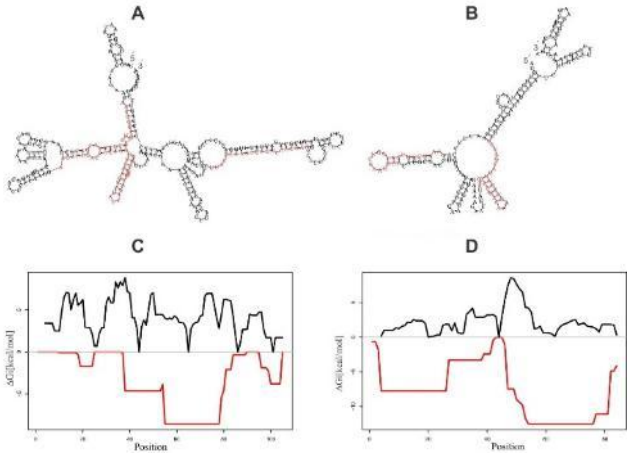

Figure - 3: Computational prediction of local secondary structure of targeted mRNA and *siRNA:mRNA* hybridization plot.

(**A)** Predicted secondary structure with lowest free energy state of PLRV-CP (300bp) mRNA. (**B)** Predicted secondary structure with lowest free energy state of ToLCNDV-AV2 (180bp) mRNA. The secondary structures were generated using RNAfold program (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). The red colored region represents the siRNAs binding sites on the predicted mRNA structures. Computational prediction of *siRNA:mRNA* hybridization plot for **(C)** PLRV-CP; (**D)** ToLCNDV-AV2 based on free interaction energy (ΔGi). The plot shows that the integration energy (red) required for *siRNA:mRNA* hybridization is significantly lower than the free energy (black) required for targeted mRNA secondary structures formation.

Table – 1: Different isolates of PLRV and ToLCNDV used for targeting conserved sequences

S. #	Isolate description	Accession #	Targeted sequence location
(A) Potato leaf roll virus (PLRV), coat protein (CP) sequences			
1	PLRV, isolate VIRUBRA 1/047, Czech Republic, 2009	EU313202	3689-3988
2	PLRV, Egypt, 2002	AY138970	3736-4035
3	PLRV, isolate 14.2, France, 2001	AF453394	3720-4019
4	PLRV, isolate PLRV-HB, China, 2009	KC456053	3737-4036
5	PLRV, isolate ASL2000, Germany, 2012	JQ346190	3720-4019
6	PLRV, isolate pLP93, Canada, 1997	D13954	3737-4036
7	PLRV, Poland, 1993	X74789	3736-4035
8	PLRV, Netherland, 1989	Y07496	3736-4034
9	PLRV, isolate GAF-318-4.2, Peru, 2016	KU586454	3737-4036
10	PLRV, CA, USA, 2014	KP090166	3737-4036
(B) Tomato leaf curl New Delhi virus (ToLCNDV), DNA-A segment (AV2) sequences			
1	ToLCNDV, isolate VIRO 840, India, 2015	KU196750	120-299
2	ToLCNDV, isolate Jam:02:44:Tom:10, Bangladesh, 2014	KM383742	120-299
3	ToLCNDV, isolate pChNDK31, India, 2012	HM007113	120-299
4	ToLCNDV, isolate Bahraich, India, 2007	EU309045	120-299
5	ToLCNDV, isolate Severe[Jessore], Bangladesh, 2005	AJ875157	120-299
6	ToLCNDV, isolate TC237, India, 2013	KF551582	120-299
7	ToLCNDV, Pakistan, 2007	EF620534	127-306
8	ToLCNDV, isolate JLX10, India, 2010	HM989845	120-299
9	ToLCNDV, isolate AH-P5, Pakistan, 2015	LN908936	120-299
10	ToLCNDV, isolate H47-7, Pakistan, 2004	AJ620187	121-300

Table – 2: Consensus sequence generated based on multiple sequence alignment

Viral specie targeting	Consensus sequence	Sequence size (bp)
Potato leafroll virus (PLRV), Coat protein	GAAGAGGAGGCAATCGCCGCTCAAGAAGAACTGGAGTTC CCCGAGGACGAGGCTCAAGCGAGACATTCGTGTTTACAA AGGACAACCTCATGGGCAACTCCCAAGGAAGTTTCACCT TCGGGCCGAGTCTATCAGACTGTCCGGCATTCAAGGATG GAATACTCAAGGCCTACCATGAGTATAAGATCACAAGCA TCTTACTTCAGTTCGTCAGCGAGGCCTCTTCCACCTCCTC CGGTTCCATCGCTTATGAGTTGGACCCCCATTGCAAAGT ATCATCCCTCCAGTCCTACGTCAACA	300
Tomato leaf curl New Delhi virus (ToLCNDV), AV2 gene	ATGTGGGATCCATTGTTGGACGAATTTCCAGAAAG CGTTCATGGTCTAAGGTGCATGCTAGCTGTAAAAT ATCTCCAAGAGATAGAAAAGAACTATTCCCCAGA CACAGTCGGGTACGATCTTGTCCGAGATCTCATTC TTGTTCTCCGAGCAAAAACTA TGGCGAAGCGACCAGCAGA	180

Table – 3: Putative siRNA sequences found within the viral most conserved sequences

S.#	Target location within mRNA	Putative siRNA sequences (5′-3′)	Antisense siRNA (3′-5′)-target mRNA (5′-3′) duplex	siRNA efficiency	RISC binding antisense score	RISC binding sense score	UPE	GC Ratio %
(A) PLRV coat protein gene (300 bp)								
1	127-147	Sense: CUAUCAGACUGUCCGGCAUUC Antisense: AUGCCGGACAGUCUGAUAGAC	Antisense: CAGAUAGUCUGACAGGCCGUA mRNA: GUCUAUCAGACUGUCCGGCAU	8.27	0.24	0.24	10.49	52.38
2	30-50	Sense: UGGAGUUCCCCGAGGACGAGG Antisense: UCGUCCUCGGGGAACUCCAGU	Antisense: UGACCUCAAGGGGCUCCUGCU mRNA: ACUGGAGUUCCCCGAGGACGA	7.76	0.02	-0.11	11.19	61.90
3	198-218	Sense: ACUUCAGUUCGUCAGCGAGGC Antisense: CUCGCUGACGAACUGAAGUAA	Antisense: AAUGAAGUCAAGCAGUCGCUC mRNA: UUACUUCAGUUCGUCAGCGAG	7.32	0.33	0.14	14.81	47.61
4	117-137	Sense: CGGGCCGAGUCUAUCAGACUG Antisense: GUCUGAUAGACUCGGCCCGAA	Antisense: AAGCCCGGCUCAGAUAGUCUG mRNA: UUCGGGCCGAGUCUAUCAGAC	6.87	0.14	0.02	13.75	57.14
5	107-127	Sense: GUUUCACCUUCGGGCCGAGUC Antisense: CUCGGCCCGAAGGUGAAACUU	Antisense: UUCAAAGUGGAAGCCCGGCUC mRNA: AAGUUUCACCUUCGGGCCGAG	6.54	0.14	0.02	13.08	57.14
(B) ToLCNDV AV2 gene (180)								
1	32-52	Sense: AGCGUUCAUGGUCUAAGGUGC Antisense: ACCUUAGACCAUGAACGCUUU	Antisense: UUUCGCAAGUACCAGAUUCCA mRNA: AAAGCGUUCAUGGUCUAAGGU	7.2	0.4	0.24	16.14	42.85
2	96-116	Sense: CCCCAGACACAGUCGGGUACG Antisense: UACCCGACUGUGUCUGGGGAA	Antisense: AAGGGGUCUGUGUCAGCCCAU mRNA: UUCCCCAGACACAGUCGGGUA	8.84	0.4	-0.11	16.95	57.14
3	102-122	Sense: ACACAGUCGGGUACGAUCUUG Antisense: AGAUCGUACCCGACUGUGUCU	Antisense: UCUGUGUCAGCCCAUGCUAGA mRNA: AGACACAGUCGGGUACGAUCU	7.82	0.24	0.24	14.21	52.38
4	33-53	Sense: GCGUUCAUGGUCUAAGGUGCA Antisense: CACCUUAGACCAUGAACGCUU	Antisense: UUCGCAAGUACCAGAUUCCAC mRNA: AAGCGUUCAUGGUCUAAGGUG	7.53	0.33	0.24	15.85	47.61

*All siRNAs were designed using pssRNAit web tool using *Solanum tuberosum* genome as reference for likelihood of off-target hybridization (http://plantgrn.noble.org/pssRNAit/).
siRNA efficiency: Efficiency denotes the effectiveness of designed siRNA to silence the targeted mRNA sequence. The efficiency range can vary from 0-10, higher the value greater silencing of submitted transcript.
UPE: Employed to calculate target accessibility of siRNA, which is represented by the energy required to open secondary structure around target site.

Discussion

Breaking host resistance through genetic variability in dominating viral isolates is a primary cause of crop failure. Resistance methodologies that rely on targeting single viral isolates have failed in the long term due to multiple viral attacks and synergisms among co-infecting pathogens (Tollenaere et al., 2016). Hence, it is necessary to engineer crops having multiple viral resistance traits to overcome this breakdown of resistance. The RNAi-mediated targeting of viral genes offers a practical approach for generating broad-spectrum resistance against multiple viruses (Hameed et al., 2017).

Mixed viral infections in potatoes, where a significant synergism amongst different viral strains was observed to cause severe disease symptoms, have been reported previously (Hameed et al., 2014). Devastating RNA viruses, along with DNA viruses such as ToLCNDV belonging to the genus *Begomovirus* have severely affected the potatoes worldwide (Usharani, 2004; Chung et al., 2013; Lamichhane and Venturi, 2015). A number of previous studies have utilized *in silico* approaches to design siRNA expression constructs that target plant viruses (Sarmah et al., 2015; Saxena et al., 2011; Sharma et al., 2015). Here in current study, we propose a computational approach to simultaneously target the dominating potato viruses (PLRV and ToLCNDV) through the generation of siRNA executable RNAi cassette.

The selection of targeted hotspots in the viral genome is a critical prerequisite for developing robust resistance, as the host siRNA silencing machinery works in a highly homology-dependent manner to silence the targeted regions (Saxena et al., 2011; Sharma et al., 2015). A subset of the complete nucleotide sequence of different isolates of PLRV and ToLCNDV was retrieved from GenBank and pairwise MSA was performed to identify the most conserved genomic regions of these viruses. A 300bp consensus sequence was generated from the highly conserved region of PLRV-CP. Similarly, a 180bp consensus sequence was generated for ToLCNDV-AV2. The selected consensus sequences were further used as template sequences in different online siRNA designing platforms to predict the functional siRNAs. A similar methodology of consensus sequence selection was adopted by Sharma et al., (2015), who identified siRNA generating hotspot sequences from the genome of geminiviruses infecting crop plants. Researchers have already defined a number of parameters as selection criteria for predicting highly efficient siRNAs (Birmingham et al., 2007; Elbashir et al., 2001). Here, we selectively utilized pssRNAit as a multi-featured program fulfilling the selection criteria to execute putative siRNAs. Similarly, Kohnehrouz and Nayeri (2016) and Sharma et al. (2015) identified pssRNAit an optimum choice for the *in silico* design of efficient siRNAs.

The selected siRNAs were further evaluated for numerous thermodynamic properties in order to predict their efficiency and probability to load into RISC silencing complexes. It is well documented that efficient siRNAs, which have a relatively high A/U nucleotide ratio at the 5′ end and relatively low G/C nucleotide ratio at the 3′ end, tend to have lower duplex structures formation and are more stable for RISC loading (Birmingham et al., 2007; Elbashir et al., 2001). We selected siRNAs fulfilling these essential thermodynamic parameters in order to predict the structure asymmetry with the highest interaction potential. The GC percentage of siRNA nucleotides is another important feature determining the silencing efficiency. A moderate range of 30-65% is considered optimum for efficient siRNAs functionality (Holen, 2005). In present study, we selected all the putative siRNAs having optimal GC percentages, which indirectly indicates the stability of siRNAs with a low chance of secondary structures formation during RISC loading. Another important feature for assessing siRNA efficiency is the *in silico* prediction of target accessibility, as it directly represents the hybridization of siRNAs to the targeted mRNA sites (Sharma et al., 2015; Birmingham et al., 2007). The mRNA secondary structure sometimes becomes inaccessible to siRNA binding due to fold-back symmetries that result in a stalled RISC (Tafer et

al., 2008). Here, we predicted the *siRNA-mRNA* hybridization plot, which demonstrated that the unpaired energy required for *siRNA*:*mRNA* hybridization is significantly lower as compared to the free energy required for targeted mRNA secondary structure formation (Figure - 3). This analysis predicts with 60-70% probability of siRNAs hybridization with mRNA targeted sites residing in hairpin loop regions (Heale et al., 2005). These results are in line with findings of Sharma et al., (2015) who also conducted a similar observation to predict the *siRNA*:*mRNA* hybridization analysis.

Off-target silencing in one of the major concerns related to RNAi-mediated approaches, as siRNAs might target off-sites in the host genome, which may result in abnormal phenotypic expression or suppression of other genetic traits (Casacuberta et al., 2015; Fellmann and Lowe, 2014; Jackson and Linsley, 2010). In order to avoid potential risks of off-target hybridization, we identified those putative siRNAs having a minimum complementarity of less than 7 continuous nucleotides with the potato genome (Xu et al., 2006). The pssRNAit program offers a selective filtering option of BLASTn analysis with reference genome in the database.

By using above mentioned computational approach, we have identified the most conserved hotspots in viral genomes for putative siRNAs synthesis during host RNAi mechanism. The current study represents the first report of designing siRNA executable sites to target both RNA and DNA viruses infecting potatoes. The *in silico* execution of potential siRNAs and prediction of their thermodynamic attributes might improve the efficacy of RNAi approach and can be further extended to engineer virus-resistant crops.

Author's contributions

AH conceived the idea and prepared the first draft of the manuscript. SF proposed the layout and conducted computational research work. AH and JIW analyzed the bioinformatics data. MN and TA provided assistance in preparing figures. The final draft of the manuscript was edited and approved by all co-authors.

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Compliance with ethical standards

Conflict of interest

The authors declare no financial or commercial conflict of interest.

Research involving human participants and/or animals

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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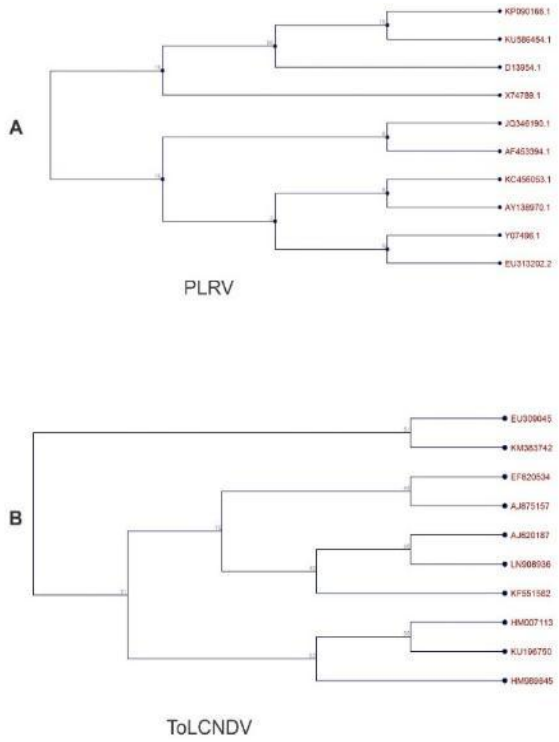

Supplementary Figure - 1: **Neighbour-joining phylogenetic dendrograms based on the alignments of all** (**A**) *Potato leafroll virus* (PLRV) and (**B**) *Tomato leaf curl New Delhi virus* (ToLCNDV) isolates used in the current study. Vertical branches are arbitrary while the horizontal branches are proportional to calculated mutation distance. Values at nodes indicate percentage bootstrap values (1000 replicates). The database accession numbers are given in each case to represent viral isolates.