Identification of siRNA generating hot spots in multiple viral suppressors to generate broad-spectrum antiviral resistance in plants

Veerendra Kumar Sharma · Nirbhay Kushwaha · Saumik Basu ·
Ashish Kumar Singh · Supriya Chakraborty

Received: 9 July 2014 / Revised: 16 September 2014 / Accepted: 22 September 2014 / Published online: 10 October 2014
© Prof. H.S. Srivastava Foundation for Science and Society 2014

Abstract Viruses are one of the most devastating plant pathogens causing severe economic losses worldwide. RNA silencing is a robust technology to knock down the expression of specific genes. This mechanism can be exploited to generate virus resistant plants through expression of the viral derived sequences. Viruses in turn have evolved to encode suppressors of RNA silencing to combat host defense. Mixed infection of plants is of common occurrence in nature and simultaneous targeting of suppressor(s) of multiple viruses offers an effective strategy. In this study, we have in silico designed siRNAs against suppressors of the two most devastating viruses of tomato, leaf curl causing tomato begomoviruses and *Cucumber mosaic virus*. Three different siRNA prediction programs were used to evaluate siRNAs generating capability of each sequence and common putative candidate siRNAs were selected fulfilling the stringent parameters. Our results indicated that in the case of each suppressor a particular region of 100–150 base pairs could be source of potent siRNAs referred as hotspots. Expression of these viral hot spots as a single construct in the plants would facilitate development of transgenic plants with a high degree of broad spectrum resistance against multiple viruses.

Keywords RNA silencing · siRNA · Viral suppressors · Mixed infection · Geminivirus · CMV

Introduction

Viruses are obligate intracellular parasites and are responsible for several devastating diseases of food, fiber and medicinal crops causing significant quantitative and qualitative losses of several crops. Tomato (*Solanum lycopersicum* L.) belongs to the *Solanaceae* family which also constitutes of several other agriculturally valuable crops. Tomato is one of the most economically important vegetable crops in the world and is being used as vegetable, sauce etc.. Several studies have revealed that this crop is highly susceptible to diverse type of viruses in natural condition (García-Cano et al. 2006). Among the different viruses infecting tomato, leaf curl causing geminiviruses and Cucumber mosaic virus (CMV) are the two major viruses imposing significant threat to successful cultivation of this crop worldwide. During mixed infections, the synergism might result in the exacerbation of disease symptoms, the increase of virus titer, and the complementation of movement defects due to host restrictions, so that either of them can spread systemically and accumulate at higher level (García-Cano et al. 2006; Chakraborty et al. 2008; Rentería-Canett et al. 2011). Geminiviruses are ssDNA viruses causing leaf curl disease in several crop plants including chilli, okra, cotton, cassava, radish, cabbage etc. (Moffat 1999; Boulton 2003; Mansoor et al. 2006; Chakraborty et al. 2008; Singh et al. 2012). During the last decade, geminiviruses have emerged as the most notorious plant pathogens causing severe crop loss worldwide. CMV is another devastating virus which infects about 1,200 plant species, causing significant economic losses in many vegetables and horticultural crops. CMV is a positive sense tripartite RNA virus which belongs to the family *Bromoviridae* (Palukaitis et al. 1992). CMV infected tomato plants exhibit stunted growth, yellowing and bushy phenotype with leaves showing shoestring phenotype. Under field conditions, plants

Electronic supplementary material The online version of this article (doi:10.1007/s12298-014-0264-0) contains supplementary material, which is available to authorized users.

V. K. Sharma · N. Kushwaha · S. Basu · A. K. Singh ·
S. Chakraborty (✉)
School of Life Sciences, Jawaharlal Nehru University,
New Delhi, India
e-mail: supriyachakrasls@yahoo.com

are infected with multiple viruses and mixed infection is a common phenomenon. The disease reaction hence generated is a consequence of the interaction between viruses belonging to same or different groups. Co-infection of a plant with two viruses has been demonstrated to produce more severe disease reaction as compared to single virus infected plants (Carr and Kim 1983; Wege and Siegmund 2007; Wege 2009; Syller 2012). Infections of crop plants with geminiviruses have been reported to be accompanied with the other devastating virus; CMV (Carr and Kim 1983).

RNA interference or post transcriptional gene silencing (PTGS) is a natural antiviral defense mechanism in plants. PTGS is a natural sequence specific mRNA degradation mechanism that is triggered by double stranded RNA (dsRNA) involving coordinate action of several proteins. Long dsRNA is cleaved by an endonuclease dicer into small interfering RNAs (siRNAs) (Bernstein et al. 2001). These nascent siRNAs are then incorporated into the RNA-induced silencing complex (RISC) and act as a guide to recognize a complementary RNA for its degradation (Hammond et al. 2000). Plant viruses with either DNA or RNA genomes are subjected to small RNA directed degradation of viral transcripts resulting in resistance against viruses. This has led to the outcome that RNA silencing mechanism can be exploited to generate resistance against viral pathogens. Pathogen derived resistance (PDR) (Sanford and Johnston 1985) strategies based on RNA silencing mechanism have been utilized to develop resistance against RNA or DNA viruses (Chen et al. 2004; Di Nicola-Negri et al. 2005; Bonfim et al. 2007; Ramesh et al. 2007; Zrachya et al. 2007; Vanderschuren et al. 2009; Patil et al. 2011). Recently, RNAi-based resistance to mixed infection of three different viruses has been reported in soybean plants expressing separate short hairpins from a single transgene (Zhang et al. 2011).

On the other hand, viral encoded proteins, called silencing suppressors (VSRs) have been evolved as one of the most potent arms against the host surveillance system. VSRs are found in almost all the plant viruses though reports of few viral suppressors from insects and mammalian viruses are also available at present. CMV encoded 2b protein is a potent suppressor of RNA silencing and inhibits RNA silencing through binding to dsRNA (Brigneti et al. 1998) and interfering with Ago mediated cleavage (Zhang et al. 2006). Three proteins namely AC2, AC4 and AV2 encoded by geminiviruses have been reported to suppress host RNA silencing with varied efficacy. AC2 protein inhibits transcriptional gene silencing through inactivation of adenosine kinase and Sucrose non fermenting 1 (SNF1) (Hao et al. 2003; Bisaro 2006), AC4 interferes with RNA silencing through binding with single stranded siRNAs (Chellappan et al. 2005) and AV2 interferes with RNA silencing by inhibiting RDR6-SGS3-siRNA mediated signal amplification (Glick et al. 2008).

Targeting these viral suppressors of RNA silencing using RNA silencing mechanism offers a promising tool to generate a high degree of resistance in plants and may provide a solution to this problem of major significance. In field condition, plants are not infected by single virus alone and targeting suppressor(s) of one particular virus will render the suppressor(s) of other viruses to inhibit RNA silencing hence making the approach non feasible. Earlier reports of *in silico* designing of putative siRNAs against viral suppressors (Saxena et al. 2011, 2013) do not provide a solution to the problem caused by mixed infection. In order to address this, we have adopted an approach of designing of highly efficient and off target filtered siRNAs using consensus sequences of suppressors of CMV and tomato infecting begomoviruses. We have used three different siRNA prediction programs (http://siRNA.wi.mit.edu/siRNA_search.cgi, <http://biodev.extra.cea.fr/DSIR/selectOT.php> and <http://plantgrn.noble.org/pssRNAit/>) to predict highly efficient siRNAs. We assume that targeting multiple suppressors of different viruses using single *ihpRNAi* construct may provide effective and broad spectrum resistance against tomato infecting viruses.

Materials and methods

Retrieval (Collection) of viral target sequences

Sequences of 18 different isolates of CMV, and 27 different tomato infecting begomoviruses were retrieved from NCBI (www.ncbi.nlm.nih.gov) database. The sequences of suppressors of CMV (2b protein), and begomoviruses (AC2, AC4, and AV2) were deduced from full length genomes.

Multiple sequence alignment of viral suppressors to generate consensus sequences

Different viral suppressor sequences were aligned and a consensus sequence for each suppressor was identified for each alignment using Genamics expression program (<http://www.genamics.com/expression/>). Multiple sequence alignment was performed to identify the most conserved regions of different suppressors among different viral isolates. The consensus sequence generated for each suppressor was further used as a query for designing highly effective putative siRNA sequences.

Designing of highly effective and virus specific siRNAs against target sequences

For designing highly effective siRNAs, we used three different siRNA designing softwares namely DSIR (<http://biodev.extra.cea.fr/DSIR/selectOT.php>) (Vert et al. 2006), siRNA at White head (http://sirna.wi.mit.edu/siRNA_search.cgi?tasto=

1012545632) (Yuan et al. 2004), and pssRNAit (<http://plantgrn.noble.org/pssRNAit/>). The consensus sequence derived from alignment of different suppressors was used as input query in above mentioned programs. Initially, siRNAs were designed using DSIR and siRNA at whitehead programs. Subsequently, the consensus sequences were used to generate siRNA using pssRNAit program. This program designs effective and specific siRNAs with genome-wide off-target gene assessment and have the feature for filtering off target siRNAs using the BLAST search against various plant species. We used this program to filter off target siRNA through a BLAST search against available tomato (*Solanum lycopersicum*) genome database.

Target secondary structure prediction and siRNA binding

Secondary structures for target sequences and siRNA binding sites were generated using Sfold (<http://sfold.wadsworth.org/cgi-bin/index.pl>) program. Consensus sequence for each suppressor was used as input query in Sfold and secondary structure with minimum free energy was selected.

Target accessibility prediction

Target accessibility of selected siRNAs was computed using RNAup program. siRNAs for each sequence were used in RNAup program to predict the favourable interaction of siRNA antisense strand with target mRNA.

Parameters for designing siRNAs

For designing highly efficient siRNAs various parameters were considered. These parameters included GC content range between 30 and 55 %, RISC binding score for sense and antisense strand, target accessibility, avoidance of four or more A, U, G and C, and filtering of off target and immunostimulatory motifs containing siRNAs.

Filtering of off target siRNAs

Initially, consensus sequence for each of viral suppressor was used to select common siRNAs that were generated by using two different programs namely DSIR, and siRNA at white head. Later, these consensus sequences were again used in pssRNAit program and siRNAs were generated using this program with a BLAST search with tomato genome to filter any off target siRNA.

Selection of effective siRNAs

Finally, the best siRNAs were selected which were common among at least two programs (but must be predicted by pssRNAit program) and fulfilling all the parameters such as

GC content, high score, target accessibility, RISC loading, lack of immunostimulatory motifs and no off targets.

Results

Retrieval of sequences and generation of consensus sequences

Nucleotide sequences of AC2, AC4 and AV2 genes were deduced from 27 different tomato infecting begomoviruses reported worldwide and CMV 2b gene sequences were deduced from 17 different CMV isolates (Supplementary Table 1 and 2). Multiple sequence alignment (MSA) of each suppressor sequence was performed using the CLC workbench program and the results of MSA were used to generate phylogenetic trees for each sequence (Supplementary fig. 1–4). Result of MSA did not provide a high degree of conservation among selected suppressor sequences due to the large number of diverse viral sequences used in the study. So we generated consensus sequences for each suppressor using Genamics expression software (<http://www.genamics.com/expression/>). Use of consensus sequence for prediction of siRNA offers advantage of broad spectrum resistance against multiple viruses.

Designing of highly efficient siRNA with filtered off targets

The consensus sequences generated for each suppressor was used as the input for the prediction of all the possible siRNAs. To obtain highly efficient siRNAs we used three different siRNA predicting softwares (DSIR, siRNA at Whitehead and pssRNAit). Out of these three programs pssRNAit has a novel feature of BLAST with different plant genomes to avoid any off target siRNA. Initially we designed siRNAs using DSIR and siRNA at Whitehead programs. All these programs have option of BLAST with the human genome to avoid any off target siRNA against human genome. To filter siRNAs targeting any tomato gene, BLAST was performed with *Solanum lycopersicum* cDNA/transcript libraries. In addition to removing off target siRNAs we further filtered siRNAs which may have immunostimulatory motifs. Next, we selected siRNAs which were assigned high rank and common to at least two programs but must be predicted by pssRNAit.

Properties of selected siRNAs

Several parameters like GC content, lack of self structure, high A/U content at 5' end of antisense strand or high G/C at the 5' end of the sense strand were considered while predicting putative siRNAs. Stretches of 4 or more A or T were avoided as it can lead to transcription termination. In this study, we have selected 30–55 % GC content range for siRNA as it has been shown that functional siRNAs tend to have a moderate to

Table 1 Potential siRNA sequences identified within the viral genomes

Gene	Position	Target sequence	Putative siRNA sequences	RISC binding Antisense score	RISC binding sense score
CMV 2b	179	UAGAUGGUUCGUGAACUGAUA	Sense strand: GAUGGUUCGUGAACUGAUA Antisense strand: UAUCAGUUCACGAACCAUCUA	1.16	-0.58
	85	CACAAGCAGAAUCGACGGGAA	Sense strand: CAAGCAGAAUCGACGGGAA Antisense strand: UCCCCGUGAUUCUGCUUGUG	1.15	-0.97
	187	UCGUGAACUGAUAGAGAUUA	Sense strand: GUGAACUGAUAGAGAUUA Antisense strand: UACAUCUCUACAGUUCACGA	-0.34	-1.57
	75	ACGAAGGUCUCACAAGCAGAA	Sense strand: GAAGGUCUCACAAGCAGAA Antisense strand: UUCUGCUUGUGAGACCUUCGU	0.59	-2.16
	74	GACGAAGGUCUCACAAGCAGA	Sense strand: GAAGGUCUCACAAGCAGAA Antisense strand: UUCUGCUUGUGAGACCUUCGU	-0.11	-0.68
	173	AUCAAGUAGAUUGGUUCGUGAA	Sense strand: CAAGUAGAUUGGUUCGUGAA Antisense strand: UUCACGAACCAUCUACUUGAU	1.37	-1.91
AC2	292	CCGUCCAAAUAACAGUUCACCC	Sense strand: CCGUCCAAAUAACAGUUCACCC Antisense strand: UUGAACUGUAUUUGGACGGUG	1.81	-2.83
	281	CGACAUAUCACCGUCCAAAU	Sense strand: CGACAUAUCACCGUCCAAA Antisense strand: UUGGACGGUGAUGAUGUCGUG	1.86	-1.85
	368	GGACGAGCUUACAGCCUCAGA	Sense strand: GGACGAGCUUACAGCCUCAGA Antisense strand: UGAGGCGUGAAGCUCGUCCAG	2.03	-1.46
	391	GGUCGUUUUCUUAAGAGUAUUU	Sense strand: GGUCGUUUUCUUAAGAGUAUU Antisense strand: AUACUCUUAAAGAAACGACCCAG	0.58	-1.20
	385	CAGACUGGUCGUUUUCUUAAGA	Sense strand: CAGACUGGUCGUUUUCUUAAGA Antisense strand: UUAAGAAACGACCAUCUCUGAG	1.63	-2.10
AV2	48	GGUGUAUGCUAGCAAUUUAAAU	Sense strand: GGUGUAUGCUAGCAAUUUAAA Antisense strand: UUAUUGCUAGCAAUACACCUA	0.48	-2.08
	148	GGCUAGAAAUUUAUGUCGAAAGC	Sense strand: GGCUAAGAAAUUUAUGUCGAAAGC Antisense strand: UUCGACAUAAUUCUAGCCCG	1.56	-1.5
	164	GAAGCGACCAGCAGAUUAUAU	Sense strand: GAAGCGACCAGCAGAUUAUA Antisense strand: UUAUUCUGCUGGUCGUCCGA	0.69	-1.11
	49	GUGUAUGCUAGCAAUUUAAAUA	Sense strand: GUGUAUGCUAGCAAUUUAAA Antisense strand: UUUAAUUGCUAGCAAUACACCU	0.2	-2.24
	47	AGGUGUAUGCUAGCAAUUUAAA	Sense strand: AGGUGUAUGCUAGCAAUUAAA Antisense strand: UAAUUGCUAGCAAUACACCUAA	0.96	-1.06
	125	CGUGAUUUUAUUUCAGUUAUU	Sense strand: CGUGAUUUUAUUUCAGUUAUU Antisense strand: UAACUGAAAAUAAUACACGAA	1.84	-1.3
	88	GACGUUUUCUCCCGAUACAUU	Sense strand: GACGUUUUCUCCCGAUACAUU Antisense strand: UGUUUCGGGAGAAUACGUCUU	0.25	-1.99
AC4	137	CCUCCGAUGUCAAGUCCUACA	Sense strand: CCUCCGAUGUCAAGUCCUACA Antisense strand: UAGGACUUGACAUCCGGGUG	2.66	-2.22
	201	CGAUGGAAGAUUCUGCUAGAGG	Sense strand: CGAUGGAAGAUUCUGCUAGAGG Antisense strand: UCUAGCAGAUUCUCCAUCCGAU	1.02	-0.28

Table 1 (continued)

Gene	Position	Target sequence	Putative siRNA sequences	RISC binding Antisense score	RISC binding sense score
	149	AGUCCUACAUGGACAAAGACG	Sense strand: AGUCCUACAUGGACAAAGACG Antisense strand: UAAUUGCUAGCAUACACCUAA	1.43	-1.43
	24	CGUGCUCAUCCAAUUCGAAGG	Sense strand: CGUGCUCAUCCAAUUCGAAGG Antisense strand: UUCGAAUUGGAUGAGCACGUG	2.44	-1.66
	113	CGAACAUUCAGGGAGCUAAAU	Sense strand: CGAACAUUCAGGGAGCUAAAU Antisense strand: UUAGCUCCUUGAAUGUUCGGA	1.40	-1.67

low GC content. Another important parameter of determining siRNA efficacy is efficiency score which is based upon several rules. To get highly efficient siRNAs we further eliminated siRNAs which were assigned an efficiency score below eight to ensure that selected siRNAs are efficient in functioning (Table 1).

Target accessibility

Designing of siRNAs based on only thermodynamic parameters does not always guarantee successful silencing of the target; the main cause of this positional effect is the presence of local mRNA structure/ secondary structure at the target site (Fig. 1). In this study we addressed this significant problem by using pssRNAit program. In pssRNAit program, RNAup program is employed to calculate target accessibility of each selected siRNA, which is represented by the energy, required to open secondary structure around the target site. The results showed that the energy required for specific interaction (ΔG_i) of the antisense strand of selected siRNAs ranges from -15 kcal/mol to 33 kcal/mol. This energy requirement is quite lower than the energy of secondary structures formed around the siRNA binding region on mRNA (Fig. 2).

Influence of guide strand on siRNA efficiency

The guide strand of siRNA plays crucial role in efficient gene silencing and thermodynamic asymmetry is fundamentally important for siRNA functions and loading into the RISC complex. The most effective siRNAs tend to have a relatively low T_m and duplex stability at 5'-end of the guide strand. Our results also show that 5'-end of the antisense strand in the case of each siRNA is A/U rich and relatively less stable compared to 5'-end of sense strand which is G/C rich. This antisense strand of each selected siRNA has a high probability to load into RISC complex. In addition, the RISC binding score assigned by the pssRNAit program to each siRNA also indicates the higher probability of antisense strand to load into the RISC complex (Table 1).

Off-target filtering

One important problem associated with in silico designed siRNAs is off target silencing of unintended host transcript. To address this problem we performed a BLAST search against human genome (DSIR, pssRNAit) and tomato genome sequence (pssRNAit) to filter off target siRNAs.

Hotspots of siRNA generation

For each suppressor sequence used in this study, we have identified a stretch of 100–200 nt (nucleotides) which we referred as hot spots for highly efficient siRNA generation.

Fig 1 Computational prediction of siRNA and corresponding target mRNA hybridization plot. **a** AV2, **b** AC2, **c** AC4 and **d** CMV2b. The plot shows that the interaction energy (ΔG_i) required for siRNA-mRNA

interaction is very low compared to the energy required for mRNA secondary structure formation

This region contains sequences, which give rise to siRNAs fulfilling all the parameters. This region extends from 75 to 190 bp for CMV2b, 100 to 220 bp for AC4, 280–415 bp for AC2 and from 40 to 200 bp for AV2.

Discussion

Viruses are obligate intracellular parasites and cause severe losses of food, fiber and medicinal crops. They invade plants and hijack host cellular machinery for successful replication and life cycle in host cells. In order to neutralize plant defense machinery of RNA silencing viruses have evolved suppressors which inhibit different steps of RNAi machinery. Targeting of viral suppressors using the RNAi mechanism offers a plausible solution to develop a high degree of broad spectrum resistance against multiple viruses.

Tomato is one of the most economically important vegetable crops worldwide and is susceptible to viruses from different taxa. Two most devastating tomato infecting viruses are tomato leaf curl viruses belonging to the family *Geminiviridae* and CMV. In natural conditions, mixed viral infection of

plants is a common phenomenon and this synergistic interaction among viruses helps both partners to avoid host defense mechanism and successfully propagate in the host. Previous reports suggest the strategy of in silico designing of siRNA against geminivirus suppressors as a tool to develop generic and broad spectrum resistance against tomato leaf curl disease (Saxena et al. 2011, 2013). The above approaches seem non feasible and impractical based on two important facts firstly, how to continuously feed these siRNAs into the field without generating transgenic plants as RNA molecules are highly labile and secondly, targeting suppressors of one virus does not offer an effective strategy as in fields single plant is infected by different viruses and the suppressor of other virus will inhibit RNA silencing. In order to answer these questions, we adopted the in silico approach of siRNA designing to target suppressors of multiple viruses which naturally occurred as mixed infection and we suggest that transgenic expression of these hot spots may provide broad spectrum transgenic resistance against multiple viruses.

We have employed consensus sequences instead of conserved sequences for prediction of siRNAs as siRNA designed using consensus sequence is likely to target broad ranges of

Fig. 2 Computational prediction of local secondary structures of target sequence and siRNA binding sites. This picture shows the predicted secondary structures of target sites sequences. Local secondary structures with the lowest free energy were generated using the fold program. The

colored region shows the binding site for siRNA guide strand on target sequences. *1a–1f* for CMV2b, *2a–2f* for AC2, *3a–3f* for AC4 and *4a–4f* for AV2

viruses compared to siRNAs designed using conserved sequences. The consensus sequences for each suppressor were used as an input query in three different programs to find out highly efficient siRNAs. We obtained several putative siRNA for each consensus sequence but in order to assure that designed siRNA are highly efficient and ensure effective silencing of target gene, we filtered siRNA with a low efficiency score. Next to find out best siRNA, we picked siRNAs which were common to at least two programs but must be predicted by pssRNAit fulfilling all the parameters as mentioned in methods section and assigned a high rank by each program.

Thermodynamic features of siRNAs are important determinants of their potency, predominantly by factors relating to RISC loading. Effective siRNAs tend to have a relatively lower duplex stability (T_m ; less stable, more A/U rich) toward the 5'-end of the strand that remains in RISC (the 'guide strand') and a relatively higher T_m (more stable, more G/C rich) toward the 5'-end of the degraded strand (the 'passenger strand') (Aza-Blanc et al. 2003). Thermodynamic asymmetry is a fundamental property of functional siRNAs and microRNAs (miRNAs) and directly correlates with strand loading during RISC assembly (Khvorova et al. 2003;

Schwarz et al. 2003; Tomari 2004). GC content of siRNAs has been shown to be another important parameter for siRNA efficacy. In general, functional siRNAs have a moderate to low GC content (30 to 55 %) and regions of high local GC content may be prone to problems with secondary structure formation which leads to stalled RISC (Holen 2005). Target site accessibility is another parameter that impacts the effectiveness of silencing (Muckstein et al. 2006; Tafer et al. 2008). Due to secondary structure formation in mRNA, some target sites are inaccessible to interact with siRNAs resulting in inhibition of RNAi (Bohula et al. 2003; Vickers et al. 2003). Our results of siRNA:mRNA hybridization analysis indicate that interaction energy required for binding of selected siRNA to the corresponding target mRNA is very low as compared to mRNA secondary structure (Fig. 1). These results explain that antisense strands of selected siRNAs are accessible to target sites lying in highly folded regions. It has been shown that siRNA efficacy predictions based upon a combination of the most probable mRNA secondary structure and siRNA duplex-

end stabilities improved the number of correct predictions from 60 to 70 % (Heale 2005).

Unintended off target silencing is a major concern while designing siRNA against any sequences as off target silencing of host genes may lead to an abnormal phenotype (Persengiev et al. 2004; Saxena et al. 2003; Scacheri 2004; Lin et al. 2005,) and is triggered by a 7 nt complementation between siRNA and off target (Xu et al. 2006). One possible way to eliminate off target effect is to use the lesser concentration of siRNAs (Semizarov et al. 2003; Persengiev et al. 2004), chemical modification of siRNA (Jackson 2006) or use of siRNAs with no homology to host genome. It is important to filter any off target siRNA suspected to target genes other than specific target. PssRNAit program (earlier siRNA scan) designs better siRNA for PTGS by minimizing off-target gene silencing in plants. In addition this program also filters siRNAs which may have an immunostimulatory motif.

We have identified the hot spots of siRNA generation in each viral sequence. These hot spots are 100 bp to 200 bp in

Fig. 3 Schematic diagram depicting the consequences of mixed infection in non transgenic and transgenic plant based on our proposed strategy. **a** In natural condition, mixed infection result in the severe symptom production in the infected plants due to cumulative effect of viral suppressors. **b** Transgenic plant harboring intron hairpin construct containing

suppressor sequences of multiple viruses. Intron hairpin construct will generate siRNA against suppressor of multiple viruses which would facilitate the sequence specific degradation of viral transcripts and inhibition of the viral multiplication resulting in broad spectrum resistance

length. The siRNAs predicted to be generated by these hot spots were the most effective siRNA in terms of fulfilling all the stringent parameters. These hot spots regions could be the prime targets of host PTGS machinery. These regions could be identified in other viral sequences and may be targeted by RNAi mechanism to generate effective broad spectrum resistance.

In case of mixed infections, different viruses code for proteins which are responsible for viral replication, transcription and movement to allow successful virus infection (Fig. 3a). Different viruses code for suppressor proteins which act on and block different steps of RNA silencing machinery of host. Thus targeting suppressor(s) of one virus is not competent enough to block infection by another virus, as the suppressor(s) encoded by the later can also block host RNAi mediated antiviral response (Fig 3a). We, therefore, propose that transgenic expression of hot spot regions identified in viral suppressors of tomato infecting begomoviruses and CMV, as single chimeric intron hairpin RNAi construct would generate highly effective siRNAs against multiples suppressors in tomato. These siRNAs would target the suppressors of both these destructive viruses through PTGS and facilitate development of broad-spectrum transgenic resistance (Fig. 3b).

This is the first report of in silico designing of highly efficient and specific siRNA against suppressors of multiple viruses to develop a high degree of broad spectrum transgenic resistance in tomato against devastating tomato leaf curl viruses and CMV using three different programs. We have firstly identified the presence of hot spots in geminivirus and CMV suppressor sequences through in silico search. This approach can be further extended to other economically important crops susceptible to multiple virus infections.

Acknowledgments We are thankful to Jawaharlal Nehru University for providing DST-PURSE grant.

Author Disclosure The authors declare that they have no competing financial interests.

References

- Aza-Blanc P, Cooper CL, Wagner K, Batalov S, Deveraux QL, Cooke MP (2003) Identification of modulators of TRAIL-induced apoptosis via RNAi-based phenotypic screening. *Mol Cell* 12:627–637
- Bernstein E, Caudy AA, Hammond SM, Hannon GJ (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409:363–366
- Bisaro DM (2006) Silencing suppression by geminivirus proteins. *Virology* 344:158–168
- Bohula EA, Salisbury AJ, Sohail M, Playford MP, Riedemann J, Southern EM, Macaulay VM (2003) The efficacy of small interfering RNAs targeted to the type 1 insulin-like growth factor receptor (IGF1R) is influenced by secondary structure in the IGF1R transcript. *J Biol Chem* 278:15991–15997
- Bonfim K, Faria JC, Nogueira EPL, Mendes ÉA, Aragão FJL (2007) RNAi-mediated resistance to *bean golden mosaic virus* in genetically engineered common bean (*Phaseolus vulgaris*). *Mol Plant Microbe* 20:717–726
- Boulton MI (2003) Geminiviruses: major threats to world agriculture. *Ann App Biol* 142:143
- Brigneti G, Voinnet O, Li WX, Ji LH, Ding SW, Baulcombe DC (1998) Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO J* 17:6739–6746
- Carr RJ, Kim KS (1983) Evidence that *bean golden mosaic virus* invades non-phloem tissue in double infections with tobacco mosaic virus. *J Gen Virol* 64:2489–2492
- Chakraborty S, Vanitharani R, Chattopadhyay B, Fauquet CM (2008) Supervirulent pseudorecombination and asymmetric synergism between genomic components of two distinct species of begomovirus associated with severe tomato leaf curl disease in India. *J Gen Virol* 89:818–828
- Chellappan P, Vanitharani R, Fauquet CM (2005) MicroRNA-binding viral protein interferes with *Arabidopsis* development. *Proc Natl Acad Sci U S A* 102:10381–10386
- Chen Y-K, Lohuis D, Goldbach R, Prins M (2004) High frequency induction of RNA-mediated resistance against *Cucumber mosaic virus* using inverted repeat constructs. *Mol Breed* 14:15–226
- Di Nicola-Negri E, Brunetti A, Tavazza M, Ilardi V (2005) Hairpin RNA-mediated silencing of *Plum pox virus* P1 and HC-Pro genes for efficient and predictable resistance to the virus. *Transgenic Res* 14: 989–994
- García-Cano E, Resende RO, Fernández-Muñoz R, Moriones E (2006) Synergistic interaction between *tomato chlorosis virus* and *tomato spotted wilt virus* results in breakdown of resistance in tomato. *Phytopathology* 96:1263–1269
- Glick E, Zrachya A, Levy Y, Mett A, Gidoni D, Belausov E, Citovsky V, Gafni Y (2008) Interaction with host SGS3 is required for suppression of RNA silencing by *tomato yellow leaf curl virus* V2 protein. *Proc Natl Acad Sci U S A* 105:157–161
- Hammond SM, Bernstein E, Beach D, Hannon GJ (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* 404:293–296
- Hao L, Wang H, Sunter G, Bisaro DM (2003) Geminivirus AL2 and L2 proteins interact with and inactivate SNF1 kinase. *Plant Cell* 15: 1034–1048
- Heale BSE (2005) siRNA target site secondary structure predictions using local stable substructures. *Nucleic Acids Res* 33:e30
- Holen T (2005) Mechanisms of RNAi: mRNA cleavage fragments may indicate stalled RISC. *J RNAi Gene Silenc* 1:21–25
- Jackson AL (2006) Position-specific chemical modification of siRNAs reduces “off-target” transcript silencing. *RNA* 12:1197–1205
- Khvorova A, Reynolds A, Jayasena SD (2003) Functional siRNAs and miRNAs Exhibit Strand Bias. *Cell* 115:209–216
- Lin X, Ruan X, Anderson MG, McDowell JA, Kroeger PE, Fesik SW, Shen Y (2005) siRNA-mediated off-target gene silencing triggered by a 7 nt complementation. *Nucleic Acids Res* 33: 4527–4535
- Mansoor S, Amrao L, Amin I, Briddon RW, Malik KA, Zafar Y (2006) First report of cotton leaf curl disease in central and southern Sindh province in Pakistan. *Plant Dis* 90:826
- Moffat AS (1999) Geminiviruses emerge as serious crop threat. *Science* 286:1835
- Muckstein U, Tafer H, Hackermuller J, Bernhart SH, Stadler PF, Hofacker IL (2006) Thermodynamics of RNA-RNA binding. *Bioinformatics* 22:1177–1182
- Palukaitis P, Roossinck MJ, Dietzgen RG, Francki RI (1992) Cucumber mosaic virus. *Adv Virus Res* 41:281–348

- Patil BL, Ogowok E, Wagaba H, Mohammed IU, Yadav JS, Bagewadi B, Taylor NJ, Kreuze JF, Maruthi MN, Alicai T et al (2011) RNAi-mediated resistance to diverse isolates belonging to two virus species involved in cassava brown streak disease. *Mol Plant Pathol* 12:31–41
- Persengiev SP, Zhu X, Green MR (2004) Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). *RNA* 10:12–18
- Ramesh SV, Mishra AK, Praveen S (2007) Hairpin RNA-mediated strategies for silencing of *tomato leaf curl virus* AC1 and AC4 genes for effective resistance in plants. *Oligonucleotides* 17:251–257
- Rentería-Canett I, Xoconostle-Cázares B, Ruiz-Medrano R, Rivera-Bustamante RF (2011) Geminivirus mixed infection on pepper plants: Synergistic interaction between PHYVV and PepGMV. *Virol J* 8:104
- Sanford JC, Johnston SA (1985) The concept of parasite-derived resistance-Deriving resistance genes from the parasite's own genome. *J Theo Biol* 113:395–405
- Saxena S, Jonsson ZO, Dutta A (2003) Small RNAs with imperfect match to endogenous mRNA repress translation. Implications for off-target activity of small inhibitory RNA in mammalian cells. *J Biol Chem* 278:44312–44319
- Saxena S, Kesharwani RK, Singh V, Singh S (2013) Designing of putative siRNA against geminiviral suppressors of RNAi to develop geminivirus-resistant papaya crop. *IJBRA* 9:3
- Saxena S, Singh N, Ranade SA, Babu SG (2011) Strategy for a generic resistance to geminiviruses infecting tomato and papaya through *in silico* siRNA search. *Virus Genes* 43:409–434
- Scacheri PC (2004) Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. *Proc Natl Acad Sci U S A* 101:1892–1897
- Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD (2003) Asymmetry in the assembly of the RNAi enzyme complex. *Cell* 115:199–208
- Semizarov D, Frost L, Sarthy A, Kroeger P, Halbert DN, Fesik SW (2003) Specificity of short interfering RNA determined through gene expression signatures. *Proc Natl Acad Sci U S A* 100:6347–6352
- Singh AK, Chattopadhyay B, Chakraborty S (2012) Biology and interactions of two distinct monopartite begomoviruses and betasatellites associated with radish leaf curl disease in India. *Virol J* 9:43
- Syller J (2012) Facilitative and antagonistic interactions between plant viruses in mixed infections. *Mol Plant Pathol* 13:204–216
- Tafer H, Ameres SL, Obernosterer G, Gebeshuber CA, Schroeder R, Martinez J, Hofacker IL (2008) The impact of target site accessibility on the design of effective siRNAs. *Nat Biotechnol* 26:578–583
- Tomari Y (2004) A protein sensor for siRNA asymmetry. *Science* 306:1377–1380
- Vanderschuren H, Alder A, Zhang P, Gruijssem W (2009) Dose-dependent RNAi-mediated geminivirus resistance in the tropical root crop cassava. *Plant Mol Biol* 70:265–272
- Vert JP, Foveau N, Lajaunie C, Vandenbrouck Y (2006) An accurate and interpretable model for siRNA efficacy prediction. *BMC Bioinforma* 7:520
- Vickers TA, Koo S, Bennett CF, Croke ST, Dean NM, Baker BF (2003) Efficient reduction of target RNAs by small interfering RNA and RNase H-dependent antisense agents. A comparative analysis. *J Biol Chem* 278:7108–7118
- Wege C (2009) Mixed infections of geminiviruses and unrelated RNA viruses or viroids in tomato: a multitude of effects with a highly probable impact on epidemiology and agriculture. In: *Crop Plant Resistance to Biotic and Abiotic Factors: Current Potential and Future Demands*, pp. 233–241
- Wege C, Siegmund D (2007) Synergism of a DNA and an RNA virus: Enhanced tissue infiltration of the begomovirus *Abutilon mosaic virus* (AbMV) mediated by *Cucumber mosaic virus* (CMV). *Virology* 357:10–28
- Xu P, Zhang Y, Kang L, Roossinck MJ, Mysore KS (2006) Computational estimation and experimental verification of off-target silencing during posttranscriptional gene silencing in plants. *Plant Physiol* 142:429–440
- Yuan B, Latek R, Hossbach M, Tuschl T, Lewitter F (2004) siRNA selection server: an automated siRNA oligonucleotide prediction server. *Nucleic Acids Res* 32:W130–W134
- Zhang X, Sato S, Ye X, Dorrance AE, Morris TJ, Clemente TE, Qu F (2011) Robust RNAi-based resistance to mixed infection of three viruses in soybean plants expressing separate short hairpins from a single transgene. *Phytopathology* 101:1264–1269
- Zhang X, Yuan YR, Pei Y, Lin SS, Tuschl T, Patel DJ, Chua NH (2006) *Cucumber mosaic virus*-encoded 2b suppressor inhibits *Arabidopsis* Argonaute1 cleavage activity to counter plant defense. *Genes Dev* 20:3255–3268
- Zrachya A, Kumar PP, Ramakrishnan U, Levy Y, Loyter A, Arazi T, Lapidot M, Gafni Y (2007) Production of siRNA targeted against TYLCV coat protein transcripts leads to silencing of its expression and resistance to the virus. *Transgenic Res* 16:385–398