# Strategy for Generic Resistance Against Begomoviruses Through RNAi

8

Nikita Shukla, Saurabh Verma, G Sunil Babu, and Sangeeta Saxena

**Abstract**

RNA interference (RNAi) is a natural gene regulatory mechanism that limits gene expression either by suppressing transcription (transcriptional gene silencing) or by promoting the sequence-specific mRNA degradation (posttranscriptional gene silencing). RNAi utilizes dsRNA molecule along with a group of proteins consisting of Argonaute (AGO), Dicer, and few RISC-associated proteins for generation of small noncoding RNAs (ncRNAs), i.e., microRNA (miRNA) and small interfering RNA (siRNA) of 21–23 nt in length which actually bind with target mRNA and regulate their gene expression. However, there is a slight difference in their mechanism of action; for instance, miRNA partially binds to target mRNA and mainly results in translational suppression, while siRNA shows complete complementarity to putative mRNA and cleaves it resulting in gene silencing. With growing evidence every day, one of the important functions of RNAi in molecular biology seems to be protection of host genome against viruses. In case of plant viruses, begomoviruses impose a serious threat to mankind as they infect several crops like tomato, cotton, papaya, etc. leading to huge economic losses. Though several physical, chemical, and transgenic strategies are in practice to provide resistance against begomoviruses, none of them have proved out to be successful. Here we propose a strategy to develop generic resistance against begomoviruses by generating small siRNAs using various in silico strategies.

Nikita Shukla and Saurabh Verma contributed equally to this work.

N. Shukla • S. Verma • G.S. Babu • S. Saxena (✉)
Department of Biotechnology, Babasaheb Bhimrao Ambedkar University, Lucknow, UP, India
e-mail: dr_sangeeta_saxena@yahoo.com



S. Saxena, A. K. Tiwari (eds.), *Begomoviruses: Occurrence and Management in Asia and Africa*, DOI 10.1007/978-981-10-5984-1_8

## 8.1 Introduction

Genus *Begomovirus* belonging to family *Geminiviridae* subgroup III (bean golden mosaic virus group) is the largest and most devastating group of plant viruses. Begomoviruses commonly found in tropical, subtropical, and warm climates, infecting mainly dicot crops, are transmitted by *Bemisia tabaci* (*Gennadius*) also known as whiteflies. These whiteflies suck the plant sap and secrete a sugary, sticky liquid that attracts several fungi and other pests. These pests in turn infest fields of crops and cause huge losses to farmers. Once these viruliferous whiteflies, i.e., carrying begomovirus, settle on leaves, they ingest phloem sap of plants and transmit virus resulting in curling, distortion, and yellowing of leaves with reduced overall plant growth.

Non-enveloped virions are 38 nm long and 22 nm in diameter, while the enveloped forms have geminate twinned appearance with icosahedral symmetry. The capsid contains 22 pentameric capsomeres, which are made up of 110 capsid proteins (CP) with single circular ssDNA in each geminate particle. The begomovirus genome is present in either a monopartite or a bipartite form. Monopartite begomoviruses have only DNA-A as a genomic component, whereas bipartite begomoviruses contain DNA-A and DNA-B, and both the DNAs are equally required for complete systemic infection. Both the components A and B are approximately 2.6 Kb in size, whereas the satellites may have genomic component ranging from 700 bp to 1.5 kb. The coding regions or open reading frames (ORFs) are present over ssDNA genome in both the virion (+) and complementary (−) sense strands. DNA-A comprises six ORFs in sense and complementary orientation. DNA-A virion (+) orientation comprises two ORFs, i.e., AV1 and AV2, which encode coat protein (CP) and precoat protein, respectively (Padidam et al. 1996), while complementary (−) sense has four ORFs, namely, AC1, AC2, AC3, and AC4. AC1 encodes very important replication-associated protein required for viral DNA replication (Hanley-Bowdoin et al. 1999), AC2 and AC3 encode transcription activator protein (TrAP) and replication enhancer protein (REn), respectively (Sunter et al. 1990; Sunter and Bisaro 1992), and AC4 is mainly reported to counteract against post-transcriptional gene silencing (Vanitharani et al. 2003; Hanley-Bowdoin et al. 2013). DNA-B component contains two proteins, namely, BC1 in sense (+) strand which encodes movement protein (MP) and BV1 in sense (−) strand encoding nuclear shuttle protein (NSP). Both the DNA components contain a common region, acting as origin of replication, and help in bidirectional transcription. On the other end, few monopartite begomoviruses such as *Tomato yellow leaf curl virus* (TYLCV), *Tomato leaf curl virus* (ToLCV), *Tomato yellow leaf curl Sardinia virus* (TYLCSV), and *Ageratum yellow vein virus* (AYVV) comprise single genomic component equivalent to DNA-A that encodes on an average six proteins, Rep, TrAP, CP, REn, C4, and V2 (Begomovirus 2017).

Monopartite begomoviruses are often found to be present along with satellite (incomplete defective genomic components) components. In this case the DNA-A component acts as a helper DNA and provides machinery to the satellite molecules for replication and transcription of its ORF. Three types of satellite DNAs

associated with begomoviruses are alphasatellite (α), betasatellite (β), and deltasatellite (δ). α- and β-satellites have circular ssDNA with single ORF that encodes *alpha-Rep* and *βC1*, respectively. Both coding regions are highly conserved and contain adenine-rich sequence regions: a hairpin containing the conserved nonanucleotide region (TAATATTAC). These α- and β-satellites coinfect with helper DNA and cause disease symptoms and devastating diseases (Briddon et al. 2003; Zhou 2013). The α-satellites have no obvious function in symptom development but to help in β-satellite replication. However, βC1 is a symptom determinant, i.e., it can suppress both transcriptional and posttranscriptional gene silencing and thus antagonize plant defense mechanisms (Cui et al. 2004, 2005). The begomoviruses and their associated satellites cause economically significant diseases in a wide range of crop plants worldwide. The δ-satellite molecules share similarity to β-satellites except that they have one more hairpin loop-containing region and are mostly noncoding in nature (Lozano et al. 2016).

Once present inside their host tissue, these viruses impose an imminent threat and are very difficult to control even through various approaches such as physical, mechanical, chemical, or even biological. Management of virus infection in commercial crops greatly affects total agricultural yield, farmers, horticulturist, etc. The control measures are not so easy as virus-infected diseases are not amenable to direct methods of intervention. Symptoms of virus-infected crops can occur right from early to late stages of plant development depending upon virus inoculum load and vector population. The first step in control of begomovirus is to stop their transmission through whiteflies by using mechanical barriers and biological predators of *Bemisia* sp. The chemical approaches though sound very attractive, but they result in heavy metal pollution in environment due to slow degradation rate of most insecticides and ever-increasing resistance against them. Considering these above facts, various transgenic approaches using pathogen-derived resistance, antisense RNA and siRNA, and other genomic intervention strategies have emerged as a necessary tool to develop begomovirus-resistant crops for sustainable crop production.

## 8.2 RNA Interference (RNAi)

Discovery of small (20–30 nt) noncoding RNA molecule is one of the most important scientific breakthroughs in the past 20 years and has contributed to a significant advancement in research areas under molecular biology. Andrew Z. Fire and Craig C. Mello shared the Nobel Prize in Physiology or Medicine in 2006 for their work on RNA interference (RNAi) (Napoli et al. 1990; Cogoni et al. 1996; Fire et al. 1998). RNAi is a novel gene regulatory mechanism in which small RNAs (sRNAs) suppress transcription via degradation of target mRNA in a sequence-specific manner earlier known as co-suppression. The suppression which occurs at transcriptional level is known as posttranscriptional gene silencing (PTGS), whereas at chromatin level, it is known as transcriptional gene silencing (TGS). The recent information on translational repression of target mRNAs suggests that even partial complementarity of miRNAs leads to translational suppression of mRNA transcript

in mammals (Meister and Tuschl 2004). PTGS involves production of 19–30 nt long sRNAs, produced from their lengthy precursor dsRNAs. These dsRNA precursors interact with silencing machinery consisting of Argonaute (AGO), Dicer, and other associated proteins of RNA-induced silencing complex (RISC). This interaction with RISC results into formation of 21–23 nt long sRNAs, and one of the strand known as guide RNA gets loaded onto RISC complex. This guides RNA-RISC complex and then binds to their target mRNA site in a homology-dependent manner resulting in cleavage of target site into smaller fragments incapable of any function. Thus, a posttranscriptional gene silencing phenomenon occurs resulting in RNA silencing (Hamilton and Baulcombe 1999; Matranga et al. 2005; MacRae et al. 2006; Siomi and Siomi 2009).

Based on the origin and nature of precursor molecule and their biological function, the sRNAs can be categorized into microRNAs (miRNAs), small interfering RNA (siRNAs), and piwi RNAs (piRNAs). piRNAs are the largest (26–31 nt) class of small noncoding RNA (sncRNA) molecules (Molecular biology select 2006; Seto et al. 2007), whereas the miRNAs and siRNAs are smaller counterparts (19–24 nt). These small regulatory RNAs are present in eukaryotes only and are derived from dsRNA; however, piRNAs are derived from long single-stranded precursor molecules (Lu et al. 2005). The biogenesis of these piRNAs is distinct from that of other sRNAs and is hypothesized to be either based on piRNA phasing or a ping-pong mechanism of generation (Brennecke et al. 2007). Both the groups of regulatory RNAs need different subsets of effector proteins, i.e., siRNA and miRNA need AGO proteins, whereas piRNA interacts with piwi proteins to mediate epigenetic and posttranscriptional gene silencing (Carmell et al. 2002; Carthew and Sontheimer 2009; Siomi and Siomi 2009; Siomi et al. 2011; Burgess 2013). Being endogenous in origin, miRNA regulates expression of host genes, whereas the exogenous origin of siRNAs allows them to protect genome integrity of host from foreign or invasive nucleic acids derived from viruses, transposons, or transgenes. In *Drosophila*, the endogenous siRNAs have been shown to provide resistance against *Drosophila* transposons; hence, the siRNAs can have both types of biogenesis mechanisms inside hosts (Chung et al. 2008; Czech et al. 2008). Exogenously supplied synthetic siRNAs or endogenously produced siRNAs are reported to regulate the expression of endogenous genes, e.g., in mice and various cell lines (Watanabe et al. 2008; Stein et al. 2011).

Transgenic approaches to develop virus-resistant plants involve introduction of viral protein-coding sequences conferring pathogen-derived resistance (PDR). Usually the mRNAs targeted are those involved in the translation of capsomeres, viral replication, suppression of host immune response, and expression of RNA-dependent RNA polymerases (RdRPs) (Abel et al. 1986; Hong et al. 1995; Ahlquist 2002). The most popular transgenic plant developed using PDR strategy targeting viral coat protein was the Hawaiian papaya resistant against *Papaya ringspot virus* (Fitch et al. 1992; Ferreira et al. 2002; Gonsalves et al. 2004). The papaya transgenics thus produced saved a huge amount of papaya produced in Hawaii thus helping farmers to incur financial gains from its export. Now we have the technical know-how about the exact mechanism of RNAi conferring virus resistance in plants and

realize that antisense RNA technology was nothing but a step into the RNAi realms. In this chapter we intend to propose a strategy to develop a generic resistance based on siRNA technique against begomoviruses infecting various crops.

## 8.3 RNAi-Based Strategies

RNAi vectors consisting of inverted repeats of siRNAs in sense and antisense orientation under the control of a strong promoter have produced mixed results in conferring resistance against begomoviruses. The precursor regions of putative siRNAs are introduced into host plants that provide significantly effective resistance against begomovirus challenge in the first generation. Eventually, the efficacy of resistance is lost or diluted as further progenies are studied. This is due to recombination, higher than critical inoculum of virus in the beginning, or due to the uncontrolled plant growth conditions (Ghoshal and Sanfaçon 2015). Though various strategies are reported for construction of RNAi vectors (Hirai and Kodama 2008), the two most popular strategies for RNAi experiments are inverted repeats containing short hairpin construct (shRNA) and artificial microRNA (amiRNA) harboring target siRNA within an miRNA backbone, thus acting like a natural miRNA (Zhou and Luo 2013).

Several studies have reported siRNA approach as a key tool to develop virus-resistant crops, e.g., transgenic *Nicotiana benthamiana* using antisense RNA technique designed against C1 (recently known as AC1) encoding Rep protein mRNA of *Tomato yellow leaf curl virus* (TYLCV). This transgenic tobacco line expressing C1 antisense RNA shows effective resistance to TYLCV through at least two generations of progeny (Bendahmane and Gronenborn 1997). A study has reported a transgenic tobacco resistant to *Cotton leaf curl virus* (CLCuV). CLCuV is a begomovirus that causes severe leaf curl symptom leading to overall economic losses to farmers in Asian subcontinent. This transgenic tobacco carries various siRNA fragments derived from CLCuV. The resistance conferred by these fragments is sustained for up to 120 days upon exposure to viruliferous whiteflies from T1 to T3 generations (Asad et al. 2003) suggesting it to be an effective resistance in case the occurrence of whiteflies overlaps this time period and thus giving a sustainable resistance approach.

Further, as one step ahead, black gram (*Vigna mungo*) infected with *Vigna mungo yellow mosaic virus* (VMYMV) is reported to recover successfully after expressing dsRNA against promoter region of viral DNA-A and established the fact that DNA of a replicating virus can also be an effective target of transcriptional gene silencing (Pooggin et al. 2003). Another study reported the recovery of *Potato spindle tuber viroid* (PSTVd) infected tomato plants using RNAi via sequence-specific degradation of viroid (Sano and Matsuura 2004). Zhang et al. (2005) developed *African cassava mosaic virus* (ACMV) resistant cassava transgenics using antisense RNA technology by targeting viral mRNAs of Rep (AC1), TrAP (AC2), and REn (AC3).

Thus, various siRNA-based resistance strategies were effective in controlling whitefly-mediated infection of begomoviruses, yet the transfer of resistance across

generations of transgenic progenies is not effective probably due to dilution of siRNA expression. Therefore, there is a need to develop effective strategy for resistant transgenic crops that are able to withstand viral attacks through many generations irrespective of initial viral titer.

Several geminiviral proteins with silencing suppressor activities have been identified which makes them potential target for RNA interference-based resistance, e.g., *Citrus tristeza virus* (CTV) resistant transgenic Mexican lime was developed by posttranscriptional gene silencing of p23, a silencing suppressor of *Citrus tristeza virus* (Fagoaga et al. 2006). In another interesting study, designing siRNA construct was reported for the first time, targeting v1 gene encoding coat protein (CP) of *Tomato yellow leaf curl virus* (TYLCV) (Zrachya et al. 2007). The transgenic tomato plants expressing siRNA did not develop disease symptoms 7 weeks postinoculation with the virus, while non-transgenic control plants developed disease symptoms within 2 weeks postinoculation. Hence, this strategy confers resistance to the TYLCV in transgenic plants and enables a good yield of flowers and fruit.

In case of cassava crop, Patil and coworkers developed RNAi-mediated resistance against different isolates of *Cassava brown streak virus* (CBSV) and *Cassava brown streak Uganda virus* (CBSUV) causing severe cassava brown streak disease (Patil et al. 2011). In this strategy, CP gene regions of the two *Potyvirus* species were targeted; the region consists of N-terminal 397 nt and C-terminal 491 nt constituting the full-length coat protein (FL-CP) gene of CBSV and CBSUV. The transgenic tobacco homozygous line expressing FL-CP region showed significant resistance (approximately 85% plants were completely resistant) against CBSUV-[UG:Nam:04] challenge, while the N-terminal and C-terminal expressing transgenic tobacco lines showed resistance in decreasing order. Therefore, the RNAi strategy appeared to be effective in case of cassava-infecting *Potyvirus* species.

Using a novel approach, designing siRNA against the most conserved region among coat protein (AV1) and replicase (AC1) genes of different isolates of geminiviruses infecting *Papaya leaf curl virus* (PLCV) and *Tomato leaf curl virus* (TLCV) was done to address generic resistance by Saxena et al. (2011). Subsequently, Saxena et al. (2013) proposed an improvised strategy to target three important genes, i.e., AV2, AC2, and AC4, which suppress plants' RNA silencing machinery. In this study, an approach to control begomovirus-infecting papaya crop was given by designing siRNA against these three suppressors using bioinformatics tools. Studies have also reported that these viral silencing suppressors may regulate functioning of the host plant with reference to resistance against the viruses by interfering with regulatory activity of some miRNAs in host gene expression (Kasschau et al. 2003; Chen et al. 2004; Bisaro 2006).

### 8.3.1 Artificial microRNA (amiRNA)

Structurally, amiRNAs span 21 nt length, generally designed by manipulation of seed region of mature miRNA sequences within double-stranded pre-miRNA. These artificial sRNAs are designed to target one or more endogenous genes in an

organism or a cell line; amiRNA precursors are generated either by PCR overlapping methods or by substitution of a natural stem loop for artificial one with restriction sites between upstream and downstream regions in the backbone of natural miRNA (Liang et al. 2012). The amiRNA sequence selection requires that there should be no mismatches within seed region (9–11 bp) though one or two mismatches are allowed near 18–21 nt positions. The hybridization energy of target and amiRNA should also favor their strong interaction leading to efficient silencing (Alvarez et al. 2006). The above parameters are not the only factors required for amiRNA-mediated silencing. Various miRNAs have been employed to introduce amiRNAs in *Arabidopsis thaliana*, e.g., miR159a, miR167b, miR169d, miR171a, miR172a, and miR319, and rice, e.g., miR528 and miR395. The use of miRNA backbone is limited, and not one miRNA can be employed for all types of silencing. Therefore, careful selection of miRNA backbone is essential for amiRNA-based strategy to be effective in viral RNA suppression (Khraiwesh et al. 2008; Tiwari et al. 2014; Carbonell et al. 2014).

The efficacy of target RNA silencing through amiRNA technology depends not only on the nature of amiRNAs but also on the accessibility of the 3′ untranslated region (UTR) of target mRNAs. The accessibility in turn depends upon the local secondary structure of the target mRNA, e.g., the tRNA-like structure found within the 3′UTR region of cucumber mosaic virus (CMV) was found to restrict target site access thus inhibiting amiRNA-RISC-mediated silencing of putative target viral RNA (Duan et al. 2008). Therefore, the target site accessibility governed by local structure at 3′UTR is as important as sequence specificity, and the target site cleavage is greatly dependent upon the interaction and thermodynamics of the small RNA-programmed RNA-induced silencing complex (siRISC) (Tafer et al. 2008; Pratt and MacRae 2009).

The concept of amiRNA with an aim to develop virus-resistant transgenic *Arabidopsis* plants was validated by modifying miR159a precursor backbone. This amiRNA backbone targets two gene silencing suppressors of viral mRNA sequence; one is P69 of *Turnip yellow mosaic virus* (TYMV) and the other is HC-Pro of *Turnip mosaic virus* (TuMV). Transgenic *A. thaliana* plants expressing amiR-P69$^{159}$ and amiR-HC-Pro$^{159}$ were resistant to TYMV and TuMV, respectively. The transgenic plants carrying the amiR-P59$^{169}$ and amiR-HC-Pro$^{159}$ within the same construct were resistant against both the viruses (Niu et al. 2006). A comparative study was conducted to evaluate efficacy of amiRNA and short hairpin RNA (shRNA) construct targeting viral silencing suppressor protein 2b derived from cucumber mosaic virus (CMV). Here, 2b gene expression was inhibited in a much more effective manner by amiRNA strategy when compared to the shRNA construct in transient assays (Qu et al. 2007). Therefore, the amiRNA technique has a potential to provide an effective and specific resistance against viral suppressors of plant RNAi machinery.

amiRNAs were studied in *Arabidopsis*, rice, wheat, maize, *Chlamydomonas reinhardtii*, etc. where they actually function like natural miRNAs (Schwab et al. 2006; Alvarez et al. 2006; Qu et al. 2007; Warthmann et al. 2008; Molnar et al. 2009; Yan et al. 2012; Fahim et al. 2012, Fahim and Larkin 2013; Xuan et al. 2015) (refer Table 8.1). These small regulatory RNAs based on miRNA precursors to

**Table 8.1** Different RNAi strategies against *Begomovirus*

| S. no. | Virus | Host | Approach | Target gene/ region | Transgenic plant | References |
|---|---|---|---|---|---|---|
| 1 | *Turnip yellow mosaic virus* (TYMV) and *Turnip mosaic virus* (TuMV) | *Brassica rapa* | Gene pyramiding | Gene silencing suppressor P69 of TYMV and HC-Pro of TuMV | *Arabidopsis thaliana* | Niu et al. (2006) |
| 2 | Cucumber mosaic virus (CMV) | *Cucumis sativus* | amiRNA mediated | Gene silencing suppressor 2b of CMV | *Nicotiana benthamiana* | Qu et al. (2007) |
| 3 | *Wheat streak mosaic virus* (WSMV) | *Triticum aestivum* | amiRNA mediated | 5′UTR, ORF pipo region of P3 cistron, P1 gene, P3 cistron upstream of pipo, Hc-Pro of WSMV genome | *Triticum aestivum* | Fahim et al. (2012) |
| 4 | *Cotton leaf curl Burewala virus* (CLCuBuV) | Cotton | amiRNA mediated | V2 gene sequence of CLCuBuV | *Nicotiana benthamiana* | Ali et al. (2013) |
| 5 | *Rice black-streaked dwarf virus* (RBSDV) | *Zea mays* | amiRNA mediated | RBSDV coding gene and gene silencing suppressor | *Zea mays* | Xuan et al. (2015) |
| 6 | *Wheat dwarf virus* (WDV) | *Triticum aestivum* | amiRNA mediated | Different conservative sequence elements of WDV strains | *Hordeum vulgare* | kis et al. (2016) |
| 7 | *Tomato yellow leaf curl virus* (TYLCV) | *Solanum licopersicum* | Antisense RNA | C1 encoded Rep protein | *Nicotiana benthamiana* | Bendahmane and Gronenborn (1997) |
| 8 | *Cotton leaf curl virus* (CLCuV) | Cotton | Antisense RNA | Rep (AC1), TrAP (AC2), and REn (AC3) | *Nicotiana benthamiana* | Asad et al. (2003) |
| 9 | *Vigna mungo yellow mosaic virus* (VMYMV) | *Vigna mungo* | hpRNA construct | Promoter sequence of VMYMV<br>209 bp long (2650–130 nt) position | *Vigna mungo* | Pooggin et al. (2003) |

(continued)

**Table 8.1** (continued)

| S. no. | Virus | Host | Approach | Target gene/ region | Transgenic plant | References |
|---|---|---|---|---|---|---|
| 10 | *African cassava mosaic virus* (ACMV) | Cassava | Antisense RNA | Rep (AC1), TrAP(AC2), and REn (AC3) | *Cassava* | Zhang et al. (2005) |
| 11 | *Citrus tristeza virus* (CTV) | *Mexican lime* | Antisense RNA | P23 | Mexican lime | Fagoaga et al. (2006) |
| 12 | *Tomato yellow leaf curl virus* (TYLCV) | *Solanum licopersicum* | siRNA construct | V1 gene encoding coat protein | *Solanum licopersicum* | Zrachya et al. (2007) |
| 13 | *Banana bunchy top virus* (BBTV) | *Banana* sp. | ihpRNA | Rep | *Banana* sp. | Shekhawat et al. (2012) |
| 14 | *Cassava brown streak virus* (CBSV) and *Cassava brown streak Uganda virus* (CBSUV) | Cassava | RNAi construct | Coat protein (CP) | *Nicotiana benthamiana* | Patil et al. (2011) |
| 15 | *Papaya leaf curl virus* (PLCuV) | *Carica papaya* | siRNA mediated | AV2, AC2, and AC4 | In silico | Saxena et al. (2013) |
| 16 | *Papaya leaf curl virus* (PLCV) and *Tomato leaf curl virus* (TLCV) | *Carica papaya*<br>*Solanum lycopersicum* | siRNA mediated | AV1 and AC1 | In silico | Saxena et al. (2013) |
| 17 | *Indian cassava mosaic virus* (ICMV) | *Jatropha curcas* | Hairpin dsRNA | Fragment1 target gene encoding 250 bp (CP/ AV1and AC5), Fragment2 250 bp (TrAP/ AC2 and Ren/ AC3), Fragment3 609 bp (Rep/ AC1 and AC4) | *Jatropha curcas* | Ye et al. (2014) |

express artificial amiRNAs are capable enough to induce silencing of putative target gene and confer virus resistance in plants.

Studies discussed above were successful experiments where the construction and introduction of artificial miRNA precursor has been one of the most effective strategy to develop broad-spectrum resistance against multiple regions of viral genes at a time, e.g., miRNA construct expressing multiple artificial miRNAs (amiRNAs) against various viral genomic regions (Bucher et al. 2006). The advantage with amiRNA strategy is that it is easier to optimize amiRNA sequences for broad-spectrum targeting and has minimal off-target effect, i.e., enhanced specificity with high efficacy.

### 8.3.2 *In Silico* Strategy for Designing Effective siRNA Target in Plants

In silico analysis of target sequence is a critical step in the development of an effective strategy against begomoviruses. The selection parameter evaluation of siRNA should be performed in such a way that ensures optimal conditions and effective targeting of the gene of interest. The previously suggested guidelines are subdivided into first-, second-, and third-generation rules that have laid the foundation for the modern and most advanced parameters followed nowadays (Liu et al. 2012).

#### 8.3.2.1 General Guidelines for a Potent siRNA Selection

The siRNA selection criterion is derived from popular studies and algorithms, which tried to validate parameters for potent siRNA prediction (Freier et al. 1986; Zecherle et al. 1996; Tuschl et al. 1999; Elbashir et al. 2002; Yu et al. 2002; Harborth et al. 2003; Khvorova et al. 2003; Reynolds et al. 2004; Ui-Tei et al. 2004; Jackson et al. 2006; Klingelhoefer et al. 2009; Wang et al. 2010; Liu et al. 2012). The following is the summary of various parameters based upon the above studies that control efficacy and specificity of the siRNA thus designed:

1. The target site must lie deep inside coding sequence or open reading frame (ORF), generally advised to start from 100 bp from the initiation codon and avoid the last 100–200 bp region.
2. Regions of high homology, i.e., 15–20 bp, tend to be more specific in silencing the gene expression of target mRNA.
3. Targets with polymorphic loci must be avoided.
4. Isoforms tend to decrease specificity; thus, the regions that are amenable to alternative splicing should be avoided.
5. The presence of secondary structure, i.e., stem loops in the siRNA sequence, decreases the RISC accessibility and hinders the formation of RISC-siRNA complex.

6. A siRNA antisense strand with low 5′ end thermodynamic energy is favorable as it eases the loading of RISC complex. Therefore, the sense and antisense strands should have a difference in binding energy.
7. Target sites with low GC content, i.e., less than 50%, have higher potential to be processed as functional siRNA regions.
8. The siRNA designed against target regions should not be having off-targets, i.e., seed region homology, with other functional mRNA sequences of the host and associated organisms, e.g., plants and their pests (helpful as well as harmful). It is also to be considered that the off-targets if present should not be a functional component of the upstream pathway or an important regulator of plant development and functions which might lead to nonspecific and insufficient handling of miRNA-like off-target effects (Schultz et al. 2011).

Elsewhere, useful guidelines for efficient designing of amiRNA-based backbone have been proposed (Vu and Do 2016). The guidelines intend to improve amiRNA design, backbone, efficiency, and specificity. However, it is impossible to design an amiRNA that fully mimics the natural miRNA function, specificity, and efficacy.

Apart from abovementioned parameters, the siRNA datasets, efficacy prediction models, and algorithms provide a useful tool to inspect the siRNA prior to its introduction into a plant. This helps to save time, resources, and labor involved in the process and enhances the probability of success.

#### 8.3.2.2 General Strategy for siRNA Designing

The *Begomovirus* replicates by forming double-stranded intermediate after entering the host nucleus. The replication starts by replication-associated protein, and here, the virus genome might undergo recombination with plant genome or any other virus present in the vicinity (in case of mixed infection). This gives rise to genetic variations that help begomoviruses to evade the siRNA machinery of host. In present context, we have seen that the begomoviruses impose an imminent threat to global crop production due to evolution of genome, thus giving rise to genetic diversity which cannot be handled by a specific siRNA approach. It is however possible to generate some resistance if we target the genetic diversity by choosing conserved sequences to design siRNAs. This strategy will help in providing a solution to more than one isolate of same species or even phylogenetically similar begomovirus species. A study proposed that if we can find out the siRNA-generating "hot spots" in the begomovirus genome, then it is quite possible to target genera of begomoviruses (Sharma et al. 2015). This seems quite possible, as an in silico analysis of various leaf curl-causing begomoviruses has yielded regions ranging from 88% to 100% conservation among phylogenetically similar isolates around the world (unpublished data).

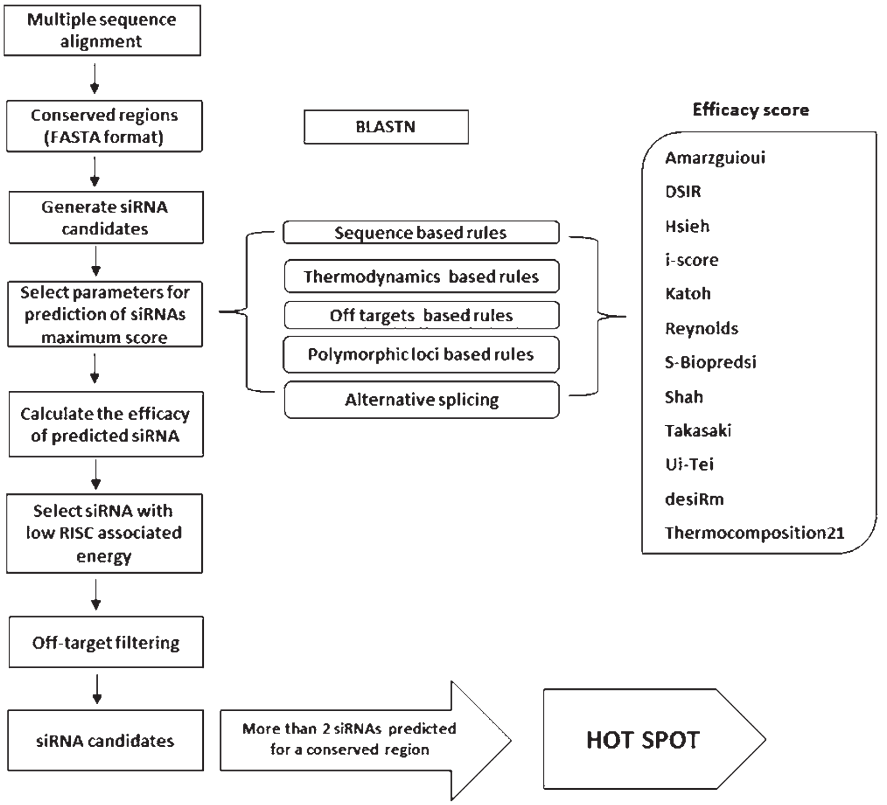



**Fig. 8.1** Outline of the siRNA designing strategy

Here we present a strategy for efficient siRNA designing that considers and fulfills the latest guidelines and rules (Fig. 8.1).

STEP 1: Sequence retrieval from various databases such as NCBI (https://www.ncbi.nlm.nih.gov), EMBL (http://www.ebi.ac.uk/embl.html), or Swiss-Prot (http://web.expasy.org/docs/swiss-prot_guideline.html).

STEP 2: Multiple sequence alignment using popular tools such as Clustal X (http://www.clustal.org/clustal2), Clustal Omega (http://www.clustal.org/omega), T-COFFEE (http://www.tcoffee.org/), and MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle). In order to find out conserved region among different viral isolates, we prefer multiple sequence alignment using software package MEGA 6.0 (http://megasoftware.net/mega.php).

STEP 3: Selection of conserved regions (85–100%) (Fig. 8.2).

STEP 4: Use of siRNA prediction tools, i.e., pssRNAit (http://plantgrn.noble.org/pssRNAit/), for prediction of siRNA target regions. Here one has to choose species according to the model being used for conducting silenc-

**Fig. 8.2** Multiple sequence alignment of the *Begomovirus* sequences using MEGA 6 package. Selection of conserved sequence regions

ing experiments. The software accordingly selects the cDNA/transcript library database for off-target effect-based analysis.

STEP 5: Analyze the parameters for the most effective siRNA-generating region. This process requires "hit and trial" approach. It is important to vary the parameters according to the sequence under study and refine them in order to achieve maximum specificity and efficacy (Figs. 8.3 and 8.4).

STEP 6: Choose siRNAs with minimal off-target score (Fig. 8.5).

STEP 7: Select hot spot regions, i.e., conserved regions producing more than two efficient siRNAs, fulfilling all rules and parameters.

Several studies have introduced large inverted hairpin siRNA construct in *N. benthamiana* without prediction of siRNAs and yet found some success in begomovirus intervention just on the basis of conservation criteria (Bucher et al. 2006; Medina-Hernandez et al. 2013). Few studies have proposed to incorporate a conserved nonanucleotide (hairpin loop) region along with large inverted repeat fragments for RNAi-based resistance due to its conserved nature (Wesley et al. 2001;

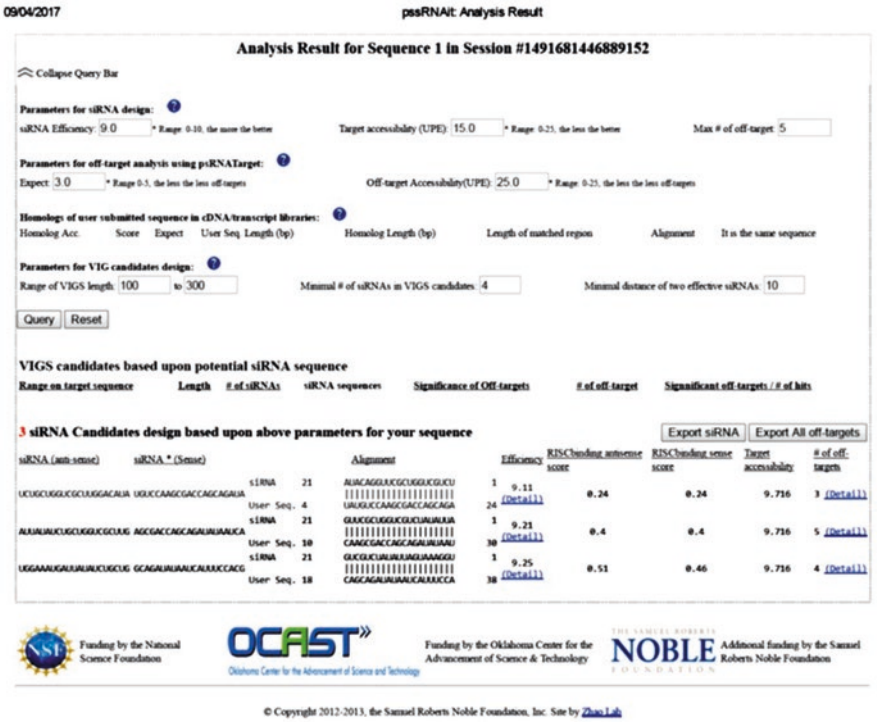



**Fig. 8.3** Designing siRNAs using pssRNAit online tool available with various parameters

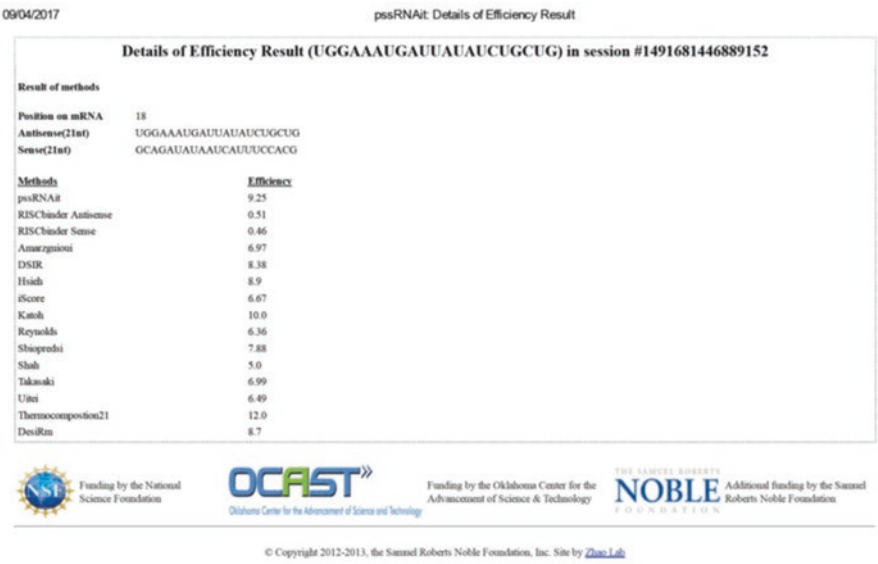
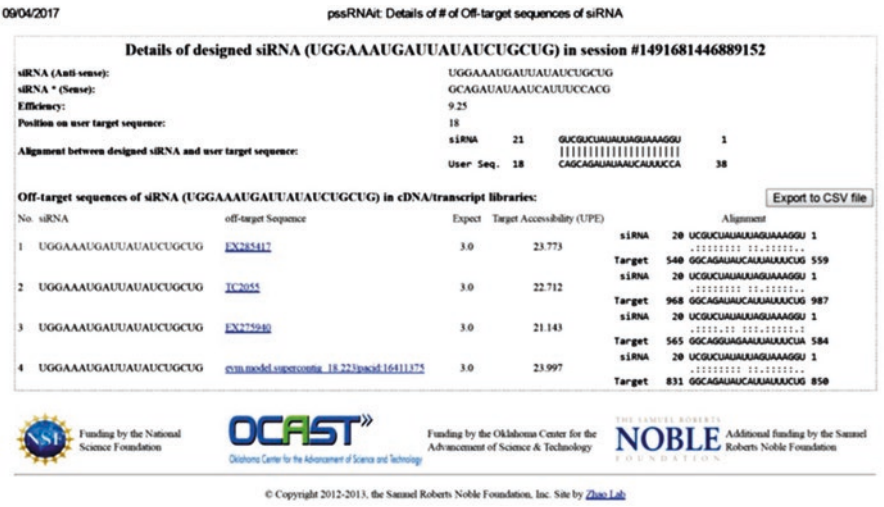



**Fig. 8.4** Details of the pssRNAit result providing various efficiency scores for a predicted siRNA (http://plantgrn.noble.org/pssRNAit/)


09/04/2017 pssRNAit: Details of # of Off-target sequences of siRNA

**Details of designed siRNA (UGGAAAUGAUUAUAUCUGCUG) in session #1491681446889152**

| | |
|---|---|
| **siRNA (Anti-sense):** | UGGAAAUGAUUAUAUCUGCUG |
| **siRNA * (Sense):** | GCAGAUAUAAUCAUUUCCACG |
| **Efficiency:** | 9.25 |
| **Position on user target sequence:** | 18 |
| **Alignment between designed siRNA and user target sequence:** | siRNA 21 GUCGUCUAUAUUAGUAAAGGU 1<br>User Seq. 18 CAGCAGAUAUAAUCAUUUCCA 38 |

**Off-target sequences of siRNA (UGGAAAUGAUUAUAUCUGCUG) in cDNA/transcript libraries:** Export to CSV file

| No. | siRNA | off-target Sequence | Expect | Target Accessibility (UPE) | Alignment |
|---|---|---|---|---|---|
| 1 | UGGAAAUGAUUAUAUCUGCUG | EX285417 | 3.0 | 23.773 | siRNA 20 UCGUCUAUAUUAGUAAAGGU 1<br>Target 540 GGCAGAUAUCAUAUAAUCUG 559 |
| 2 | UGGAAAUGAUUAUAUCUGCUG | TC2055 | 3.0 | 22.712 | siRNA 20 UCGUCUAUAUUAGUAAAGGU 1<br>Target 968 GGCAGAUAUCAUAUAAUCUG 987 |
| 3 | UGGAAAUGAUUAUAUCUGCUG | EX275940 | 3.0 | 21.143 | siRNA 20 UCGUCUAUAUUAGUAAAGGU 1<br>Target 565 GGCAGGUAGAAUUAUAUCUA 584 |
| 4 | UGGAAAUGAUUAUAUCUGCUG | evm.model.supercontig_18.223\|pacid:16411375 | 3.0 | 23.997 | siRNA 20 UCGUCUAUAUUAGUAAAGGU 1<br>Target 831 GGCAGAUAUCAUAUAAUCUG 850 |

Funding by the National Science Foundation

Funding by the Oklahoma Center for the Advancement of Science & Technology

Additional funding by the Samuel Roberts Noble Foundation



**Fig. 8.5** Details of the predicted off-targets for one of the siRNA

Khatoon et al. 2016). Therefore, the strategy of introducing hot spots rather than a single specific siRNA has an advantage over the later strategy.

## 8.4 Conclusion

RNAi being a popular tool to downregulate the gene expression at posttranscriptional level has helped in the development of various strategies that succeeded in conferring some amount of resistance against begomoviruses. Yet, the suppression of resistance is imminent in case of begomoviruses due to their recombination-dependent replication and RNAi suppression components that antagonize the plant immune response and bypass the siRNA-based silencing. Therefore, no strategy could be a complete solution for the global problem of begomovirus diseases; still, the generic solution for a particular crop in a specific region could be a possible solution until the virus isolate evolves itself into a new recombinant. Here, we have discussed few aspects of RNAi and the basis of siRNA-based strategies employed in the past in an attempt to develop begomovirus-free transgenic crops for sustainable food resources and global food security. The strategy presented here may not be the ideal solution but provides a perspective for researchers to pursue research in the very area in order to develop generic resistance against begomoviruses.

## References

Abel PP, Nelson RS et al (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232(4751):738–743

Ahlquist P (2002) RNA dependent RNA polymerases, viruses and RNA silencing. Science 296:1270–1274

Ali I, Amin I et al (2013) Artificial microRNA-mediated resistance against the monopartite begomovirus cotton leaf curl Burewala virus. Virol J 10(1):231

Alvarez JP, Pekker I et al (2006) Endogenous and synthetic microRNAs stimulate simultaneous, efficient and localized regulation of multiple targets in diverse species. Plant Cell 18:1134–1151

Asad S, Haris WAA et al (2003) Transgenic tobacco expressing geminiviral RNAs are resistant to the serious viral pathogen causing cotton leaf curl disease. Arch Virol 148:2341–2352

Begomovirus V (2017) http://viralzone.expasy.org/all_by_species/111.html. Accessed 13 Feb 2017

Bendahmane M, Gronenborn B (1997) Engineering resistance against tomato yellow leaf curl virus (TYLCV) using antisense RNA. Plant Mol Biol 33(2):351–357

Bisaro DM (2006) Silencing suppression by geminivirus proteins. Virology 344(1):158–168

Brennecke J, Aravin AA et al (2007) Discrete small RNA-generating loci as master regulators of transposon activity in drosophila. Cell 128(6):1089–1103

Briddon RW, Bull SE, Amin I, Idris AM et al (2003) Diversity of DNA beta; a satellite molecule associated with some monopartite begomoviruses. Virology 312(1):106–121

Burgess DJ (2013) Small RNAs: defining piRNA expression. Nat Rev Genet 14:301

Bucher E, Lohuis D et al (2006) Multiple virus resistance at a high frequency using a single transgene construct. J Gen Virol 87:3697–3701

Carbonell A, Takeda A et al (2014) New generation of artificial MicroRNA and synthetic trans-acting small interfering RNA vectors for efficient gene silencing in *Arabidopsis*. Plant Physiol 165:15–29

Carmell M, Xuan Z, Zhang M, Hannon G (2002) The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. Genes Dev 16(21):2733–2742

Carthew RW, Sontheimer EJ (2009) Origins and mechanisms of miRNAs and siRNAs. Cell 136(4):642–655

Chen J, Li WX et al (2004) Viral virulence protein suppresses RNA silencing-mediated defense but upregulates the role of microRNA in host gene expression. Plant Cell 16:1302–1131

Chung WJ, Okamura K et al (2008) Endogenous RNA interference provides a somatic defense against *Drosophila* transposons. Curr Biol 18(11):795–802

Cogoni C, Irelan JT et al (1996) Transgene silencing of the AL-1 gene in vegetative cells of *Neurospora* is mediated by a cytoplasmic effector and does not depend on DNA-DNA interactions or DNA methylation. EMBO J 15(12):3153–3163

Cui XF, Tao XR et al (2004) A DNA b associated with tomato yellow leaf curl China virus is required for symptom induction. J Virol 78(24):13966–139744

Cui XF, Li YQ, Hu DW, Zhou XP (2005) Expression of a begomoviral DNA b gene in transgenic *Nicotiana* plants induced abnormal cell division. J Zhejiang Univ Sci B 6(2):83–86

Czech B, Malone CD et al (2008) An endogenous small interfering RNA pathway in *Drosophila*. Nature 453(7196):798–802

Duan CG, Wang CH et al (2008) Artificial microRNAs highly accessible to targets confer efficient virus resistance in plants. J Virol 82(22):11084–11095

Elbashir SM, Harborth J et al (2002) Analysis of gene function in somatic mammalian cells using small interfering RNAs. Methods 26:199–213

Fagoaga C, Lopez C et al (2006) Post-transcriptional gene silencing of the p23 silencing suppressor of citrus tristeza virus confers resistance to the transgenic Mexican lime. Plant Mol Biol 60(2):153–165

Fahim M, Larkin PJ (2013) Designing effective amiRNA and multimeric amiRNA against plant viruses. Methods Mol Biol 942:357–377
Fahim M, Millar AA et al (2012) Resistance to wheat streak mosaic virus generated by expression of an artificial polycistronic microRNA in wheat. Plant Biotechnol J 10(2):150–163
Ferreira SA, Pitz KY et al (2002) Virus coat protein transgenic papaya provides practical control of papaya ringspot virus in Hawaii. Plant Dis 86:101–105
Fitch MMM, Manshardt RM et al (1992) Virus resistant papaya derived from tissues bombarded with the coat protein gene of papaya ringspot virus. BioTechnol 10:1466–1472
Fire A, Xu S et al (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391(6669):806–811
Freier SM, Kierzek R, Jaeger JA et al (1986) Improved free-energy parameters for predictions of RNA duplex stability. Proc Natl Acad Sci 83:9373–9377
Ghoshal B, Sanfacon H (2015) Symptom recovery in virus-infected plants: revisiting the role of RNA silencing complex. Virology 479–480:167–179
Gonsalves C, Lee DR, Gonsalves D (2004) Transgenic virus resistant papaya: from hope to reality for controlling papaya ring spot virus in Hawaii. APSnet Features. doi:10.1094/APSnetFeature-2004-0804. http://www.apsnet.org/online/feature/rainbow
Hamilton A, Baulcombe D (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286(5441):950–952
Hanley-Bowdoin L, Settlage SB et al (1999) Geminiviruses: models for plant DNA replication transcription and cell cycle regulation. CRC Crit Rev Plant Sci 18:71–106
Hanley-Bowdoin L, Bejarano ER et al (2013) Geminiviruses: masters at redirecting and reprogramming plant processes. Nat Rev Microbiol 11(11):777–788
Harborth J, Elbashir SM, Vandenburgh K et al (2003) Sequence, chemical and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing. Anitisense Nucleic Acid Drug Dev 13(2):83–105
Hirai S, Kodama H (2008) RNAi vectors for manipulation of gene expression in higher plants. Open Plant Sci J 2:21–30
Hong Y, Levay K et al (1995) A potyvirus polymerase interacts with the viral coat protein and VPg in yeast cells. Virology 214(1):159–166
Jackson AL, Burchard J, Leake D et al (2006) Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing. RNA 12:1197–1205
Kasschau KD, Xie Z et al (2003) P1/HC-Pro, a viral suppressor of RNA silencing, interferes with *Arabidopsis* development and miRNA function. Dev Cell 4:205–217
Khatoon S, Kumar A et al (2016) RNAi- mediated resistance against cotton leaf curl disease in elite Indian cotton (*Gossypium hirsutum*) cultivar 'Narasimha'. Virus Genes 52(4):530–553
Khvorova A, Reynolds A, Jayasena SD (2003) Functional siRNAs and miRNAs exhibit strand bias. Cell 115:209–216
Khraiwesh B, Ossowski S et al (2008) Specific gene silencing by artificial MicroRNAs in *Physcomitrella patens*: an alternative to targeted gene knockouts. Plant Physiol 148(2):684–693
Kis A, Tholt G et al (2016) Polycistronic artificial miRNA-mediated resistance to wheat dwarf virus in barley is highly efficient at low temperature. Mol Plant Pathol 17(3):427–437
Klingelhoefer JW, Moutsianas L, Holmes C (2009) Approximate Bayesian feature selection on a large meta-dataset offers novel insights on factors that effect siRNA potency. Bioinformatics 25:1594–1601
Liang G, He H et al (2012) A new strategy for construction of artificial miRNA vectors in *Arabidopsis*. Planta 235:1421–1429
Liu Q, Zhou H et al (2012) Reconsideration of in-silico siRNA design based on feature selection: a cross-platform data integration perspective. PLoS One 7(5):e37879
Lozano G, Trenado HP et al (2016) Characterization of non-coding DNA satellites associated with Sweepoviruses (genus Begomovirus, Geminiviridae)- definition of a distinct class of Begomovirus-associated satellites. Front Microbiol 7:162
Lu C, Tej SS et al (2005) Elucidation of the small RNA component of the transcriptome. Science 309(5740):1567–1569

MacRae IJ, Zhou K et al (2006) Structural basis for double-stranded RNA processing by Dicer. Science 311(5758):195–198
Matranga C, Tomari Y et al (2005) Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. Cell 123(4):607–620
Medina-Hernandez D, Rivera-Bustamante R et al (2013) Effects and effectiveness of two RNAi constructs for resistance to pepper golden mosaic virus in *Nicotiana benthamiana* plants. Virus 5:2931–2945
Meister G, Tuschl T (2004) Mechanisms of gene silencing by double-stranded RNA. Nature 431(7006):343–349
Molecular biology select (2006) Cell 126(2): 223–225. Accessed 12 Dec 2016
Molnar A, Bassett A et al (2009) Highly specific gene silencing by artificial microRNAs in the unicellular alga *Chlamydomonas reinhardtii*. Plant J 58(1):165–174
Napoli C, Lemieux C, Jorgensen R (1990) Introduction of a chimeric Chalcone synthase gene into *Petunia* results in co-suppression of homologous genes in trans. Plant Cell 2(4):279–289
Niu QW, Lin SS, Reyes JL et al (2006) Expression of artificial microRNAs in transgenic *Arabidopsis thaliana* confers virus resistance. Nat Biotechnol 24:1420–1428
Padidam M, Beachy RN, Fauquet CM (1996) The role of AV2 ('precoat') and coat protein in viral replication and movement in tomato leaf curl geminivirus. Virology 224(2):390–404
Patil BL, Ogwok E et al (2011) RNAi-mediated resistance to diverse isolates belonging to two virus species involved in cassava brown streak disease. Mol Plant Pathol 12(1):31–41
Pooggin M, Shivprasad PV et al (2003) RNAi targeting of DNA virus in plants. Nat Biotechnol 21:131–132
Pratt AJ, MacRae IJ (2009) The RNA-induced silencing complex: a versatile gene-silencing machine. J Biol Chem 284(27):17897–17901
Qu J, Ye J, Fang R (2007) Artificial MicroRNA-mediated virus resistance in plants. J Virol 81(12):6690–6699
Reynolds A, Leake D, Boese Q et al (2004) Rational siRNA design for RNA interference. Nat Biotechnol 22:326–330
Sano T, Matsuura Y (2004) Accumulation of small interfering RNAs characteristic of RNA silencing precedes recovery of tomato plants from severe symptoms of potato spindle tuber viroid infection. J Gen Plant Pathol 70(1):50–53
Saxena S, Kesharwani RK, Singh V (2013) Designing of putative *siRNA* against geminiviral suppressors of RNAi to develop geminivirus-resistant papaya crop. IJBRA 9(1):3–12
Saxena S, Singh N et al (2011) Strategy for a generic resistance to geminiviruses infecting tomato and papaya through in silico siRNA search. Virus Genes 43:409–434
Schultz N, Marenstein DR et al (2011) Off-target effects dominate a large-scale RNAi screen for modulators of the TGF-β pathway and reveal microRNA regulation of TGFBR2. Silence 2:3
Schwab R, Ossowski et al (2006) Highly specific gene silencing by artificial microRNAs in *Arabidopsis*. Plant Cell 18(5):1121–1133
Seto AG, Kingston RE, Lau NC (2007) The coming age for Piwi proteins. Mol Cell 26(5):603–609
Sharma VK, Kushwaha N et al (2015) Identification of siRNA generating hot spots in multiple viral suppressors to generate broad-spectrum antiviral resistance in plants. Physiol Mol Biol Plants 21(1):9–18
Shekhawat UKS, Ganapathi TR, Hadapad AB (2012) Transgenic banana plants expressing small interfering RNAs targeted against viral replication initiation gene display high-level resistance to banana bunchy top virus infection. J Gen Virol 93(8):1804–1813
Siomi H, Siomi MC (2009) On the road to reading the RNA-interference code. Nature 457(7228):396–404
Siomi MC, Sato K, Pezic D, Aravin AA (2011) PIWI-interacting small RNAs: the vanguard of genome defense. Nat Rev Mol Cell Biol 12:246–258
Stein DA, Perry ST, Buck MD (2011) Inhibition of dengue virus infections in cell cultures and in AG129 mice by a small interfering RNA targeting a highly conserved sequence. J Virol 85(19):10154–10166

Sunter G, Hartitz MD et al (1990) Genetic analysis of tomato golden mosaic virus: ORF AL2 is required for coat protein accumulation while ORF AL3 is necessary for efficient DNA replication. Virology 179(1):69–77
Sunter G, Bisaro DM (1992) Transactivation of geminivirus AR1 and BR1 gene expression by the AL2 gene product occurs at the level of transcription. Plant Cell 4(10):1321–1331
Tafer H, Ameres SL et al (2008) The impact of target site accessibility on the design of effective siRNAs. Nat Biotechnol 26(5):578–583
Tiwari M, Sharma D, Trivedi PK (2014) Artificial microRNA mediated gene silencing in plants: progress and perspectives. Plant Mol Biol 86(1):1–18
Tuschl T, Zamore PD, Lehmann R et al (1999) Targeted mRNA degradation by double-stranded RNA invitro. Genes Dev 13:3191–3197
Ui-Tei K, Naito Y, Takahashi F et al (2004) Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. Nucleic Acids Res 32:936–948
Vanitharani R, Chellappan P, Fauquet CM (2003) Short interfering RNA-mediated interference of gene expression and viral DNA accumulation in cultured plant cells. Proc Natl Acad Sci U S A 100(16):9632–9636
Vu TV, Do VN (2016) Customization of artificial microRNA design. In: Springer protocol. Methods in molecular biology 1509. pp 235–243
Wang L, Huang C, Yang JY (2010) Predicting siRNA potency with random forests and support vector machines. BMC Genomics 11(Suppl.3):S2
Warthmann N, Chen H et al (2008) Highly specific gene silencing by artificial miRNAs in rice. PLoS One 3(3):e1829
Watanabe T, Totoki Y et al (2008) Endogenous siRNAs from naturally formed dsRNAs regulate transcripts in mouse oocytes. Nature 453(7194):539–543
Wesley SV, Helliwell CA et al (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J 27(6):581–590
Xuan N, Zhao C et al (2015) Development of transgenic maize with anti-rough dwarf virus artificial miRNA vector and their disease resistance. Shen Wu Gong Cheng Xue Bao 31(9):1375–1386
Yan F, Lu Y et al (2012) A simplified method for constructing artificial microRNAs based on the osa-MIR528 precursor. J Biotechnol 60:146–150
Ye J, Qu J et al (2014) Engineering Geminivirus resistance in *Jatropha curcas*. Biotechnol Biofuels 7:149. doi:10.1186/s13068-014-0149-z
Yu JY, DeRuiter SL, Turner DL (2002) RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. Proc Natl Acad Sci 99:6047–6052
Zecherle GN, Whelen S, Hall BD (1996) Purines are required at the 5′ends of newly initiated RNAs for optimal RNA polymerase III gene expression. Mol Cell Biol 16:5801–5810
Zhang P, Vanderschuren H et al (2005) Resistance to cassava mosaic disease in transgenic cassava expressing antisense RNAs targeting virus replication genes. Plant Biotechnol J 3:385–397
Zhou X (2013) Advances in understanding Begomovirus satellites. Annu Rev Phytopathol 51:357–381
Zhou M, Luo H (2013) MicroRNA mediated gene regulation: potential applications for plant genetic engineering. Plant Mol Biol 83:59–75
Zrachya A, Kumar PP et al (2007) Production of siRNA targeted against TYLCV coat protein transcripts leads to silencing of its expression and resistance to the virus. Transgenic Res 16(3):385–398