

REVIEW

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## Advances, Challenges and Prospects in Small RNA Mediated Approaches of Virus Resistance in Plants

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**Abstract:** RNA interference (RNAi) is a mechanism of small RNA-guided regulation of gene expression in which small RNAs inhibit the expression of genes with complementary nucleotide sequences. RNAi has emerged as a powerful modality for battling challenging viruses and provides a natural defense against viral pathogens. In plants, RNAi has been successfully used to express cognate dsRNAs for viral transcripts in order to initiate the process of viral gene silencing. Despite the wide applicability of RNAi for achieving viral resistance, there are some challenges and constraints that must to be addressed to develop RNAi as a more effective tool for virus resistance. The present review provides an update on different approaches of using RNAi for virus resistance development in various crop plants. The factors influencing RNAi-mediated virus resistance and the major constraints are also discussed in detail.

**Keywords:** RNAi, dsRNA, siRNA, miRNA, VSRs, vsiRNAs

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## Introduction

Viruses are important biotic threats to plants worldwide. Limited sources of natural resistance warrant the development of novel resistance sources. In mammals, extraordinary advances have been made towards understanding the intricate workings of the antibody-based cellular immune response. However, it was unclear whether plants possessed mechanisms to immunize or vaccinate their cells until the discovery of RNA interference (RNAi) or RNA silencing.

RNA silencing is a versatile, complex gene regulation and defense mechanism targeting parasitic or endogenous RNA in a highly sequence-specific manner. These evolutionarily conserved processes are now known to be operative in most, if not all, eukaryotic organisms.<sup>1</sup> RNA silencing operates through a set of core reactions triggered by dsRNA, which is processed into 21–24 nucleotide RNA duplexes by the RNase III enzyme Dicer and its homologs.<sup>2</sup> These factors in turn mediate multiple regulatory and defense functions in cells.<sup>3</sup> Two main types of RNA molecules have the potential to serve as a trigger of RNA silencing. Small interfering RNAs (siRNAs) have been implicated in a variety of processes, including defense against viruses, establishment of heterochromatin, silencing of transposons and transgenes, and post-transcriptional regulation of genes.<sup>4</sup> Micro RNAs (miRNAs) are small endogenous RNAs that regulate gene expression in plants and animals. If RNAi was simply a novel mechanism of gene regulation, then it would still be an amazing addition to our understanding of molecular mechanisms of life; however, the importance of RNAi extends beyond its theoretical implications. Indeed, this discovery has led to the redefining molecular concepts of plant virus defense mechanisms and has provided clues to the mechanisms of RNA-based, rather than protein (antibody)-based cellular immune responses.

In the past few years, RNAi has become one of the most exciting discoveries of molecular biology due to its high specificity, accuracy, and heritability. Technologies based on RNAi pathways have shown its potential in a very short amount of time and in wide range of field applications.<sup>5–7</sup> The most important application of RNAi in plant biology is achieving virus resistance in a number of agricultural and horticultural crops. In fact, the first biological

function established for RNA silencing was as an antiviral mechanism in plants.<sup>8–10</sup> It was subsequently established that virus infection induces RNAi in the host, which then targets viral RNAs to confer virus resistance.<sup>11,12</sup>

The theory that RNAi is an antiviral mechanism in plants was further supported by two additional lines of evidence. First, mutants carrying loss-of-function mutations in essential silencing pathway genes, such as *rdr6*, *ago1*, and *dcl2*, showed enhanced disease susceptibility to viral infection.<sup>13–15</sup> Second, essential virulence factors of many plant RNA and DNA viruses are suppressors of RNAi.<sup>16–18</sup> In subsequent reports, it was demonstrated that virus-specific siRNAs of both positive and negative polarities accumulate in plants infected with viruses,<sup>19,20</sup> thereby establishing that viral RNAs induce the RNAi pathway and serve as a substrate of Dicer.<sup>21</sup>

RNAi technology for the generation of viral resistant plants was first demonstrated in *Potato virus Y* (PVY), wherein complete immunity to the virus was reported in potato plants after simultaneous expression of both the sense and antisense transcripts of the viral helper-component proteinase (*HC-Pro*) gene.<sup>10</sup> This finding paved the way for deploying RNAi-mediated resistance against several other viruses (Table 1). Here, we review the present understanding of small RNA approaches for the development of virus resistance in plants. Various construct designing strategies and factors are discussed, starting with what has been learned from different reports about the fundamentals of RNAi-mediated virus resistance traits. In addition, we also highlight the major constraints in achieving the goal of antiviral function of small RNAs.

## Small RNAs: Key Players in the RNAi Pathway

Small RNAs are key mediators of RNA silencing-related pathways in plants and other eukaryotic organisms. Endogenous or exogenous small RNAs can either guide post-transcriptional gene silencing by mRNA cleavage/degradation/translational inhibition or guide transcriptional gene silencing by DNA methylation and chromatin modifications. Small RNA molecules in plants, such as small interfering RNA (siRNA) and microRNA (miRNA), require Dicer enzyme for processing. Although small RNAs share common features, there are some differences in

**Table 1.** A list of plant viruses targeted for resistance development using RNAi with different approaches and transgenes.

Transgenic crop plant/virus	Transgene	Type of RNAi construct	Reaction	Reference
<b>Wheat</b>				
<i>Wheat streak mosaic virus</i>	<i>Nla</i>	Hairpin RNA	Complete resistance	147
<b>Cassava</b>				
<i>African cassava mosaic virus</i>	<i>AC1</i>	Homologous hairpin double-strand RNAs	Immunity	148
<i>Cassava brown streak Uganda virus</i>	<i>cp</i>	Self-complementary hairpin RNA	Resistance	62
<b>Tobacco</b>				
<i>Cucumber mosaic virus</i>	<i>3'UTR</i>	Hairpin RNA	Delayed resistance, recovery phenotypes	138
<i>Tobacco mosaic virus and Cucumber mosaic virus</i>	<i>mp + Rep</i>	Intron-hairpin-RNA	Resistance	69
<i>Cucumber mosaic virus</i>	<i>2b</i>	amiRNA	Effective resistance	76
<i>Cassava brown streak virus and Cassava brown streak Uganda virus</i>	<i>cp</i>	RNAi constructs	Full-length construct showing highest resistance	149
<i>Citrus tristeza virus</i>	<i>p23 + 3'UTR</i>	RNAi construct	Resistance	150
<i>Plum pox virus</i>	PPV RNA genome (from base 134 to 330)	Self-complementary hairpin RNAs	Systemic resistance	151
<i>Citrus psorosis virus</i>	<i>cp</i> and <i>54 K</i> genes	Self-complementary hairpin RNA	Varied resistance	152
<i>Cucumber green mottle mosaic virus</i>	<i>cp</i>	Inverted repeat RNA	Strong and efficient resistance	55
<i>Cucumber mosaic virus</i>	3' portion of the CMV RNA3 genome containing <i>cp</i> gene	Inverted repeat RNA	Varied resistance	39
<i>Grapevine Fan leaf virus</i>	<i>mp</i>	Inverted repeat RNA	Varied resistance	59
<i>Pepper mild mottle virus</i>	–	Self-complementary hairpin RNA	Resistance	60
<i>Potato virus Y and Potato virus X</i>	<i>HC-Pro + p25</i>	amiRNA	Resistance	78
<b>Tobacco and Prunus domestica</b>				
<i>Plum pox virus</i>	<i>cp</i>	Intron-hairpin-RNA	Resistance	153
<b>Common bean</b>				
<i>Bean golden mosaic virus</i>	<i>AC1</i>	Intron-hairpin	High resistance	71
<b>Rice</b>				
<i>Rice tungro bacilliform virus</i>	<i>ORF IV</i>	DNA encoding in sense as well as in anti-sense orientation	Variable resistance responses	50
<i>Rice dwarf virus</i>	<i>Pns12</i> and <i>Pns4</i>	RNAi constructs	<i>Pns12</i> -specific RNAi constructs- strongly resistant and <i>Pns4</i> -specific RNAi constructs- less apparent resistance	94
<b>Tomato</b>				
<i>Tomato yellow leaf curl virus</i>	Replication associated protein gene ( <i>C1</i> )	Intron-hairpin	Immunity	65
<i>Potato spindle tuber viroid</i>	–	Hairpin RNA	Resistance	154
<i>Tomato leaf curl virus</i>	<i>AC1</i> and <i>AC4</i>	Hairpin RNA	Resistance	155
<i>Tomato leaf curl virus</i>	<i>AC4</i>	RNAi constructs	Variable resistance	156

(Continued)



Table 1. (Continued)

Transgenic crop plant/virus	Transgene	Type of RNAi construct	Reaction	Reference
<b>Sugarbeet</b>				
<i>Beet necrotic yellow vein virus</i>	Rep	Inverted repeat RNA	Resistance	53
<b>Soybean</b>				
<i>Soybean dwarf virus</i>	cp	Inverted repeat RNA	Resistance	54
<b>Barley</b>				
<i>Barley yellow dwarf virus</i>	Polymerase gene	Self-complementary hairpin RNA	Resistance	38
<b>Potato</b>				
<i>Potato virus Y</i>	cp	Self-complementary hairpin RNA	Resistance	61
<b>Sweet potato</b>				
<i>Sweetpotato chlorotic stunt virus</i>	Rep	Intron-spliced hairpin	Mild or no symptoms, reduced accumulation of virus	157
<b>Sweet orange</b>				
<i>Citrus psorosis virus</i>	cp, 54 K and 24 K genes	Intron-hairpin-RNA	Coat protein: fully resistance 54 K or 24 K: partially resistant	70
<b>Maize</b>				
<i>Sugarcane mosaic virus</i>	cp	Direct delivery by spraying	Resistance	82
<i>Maize dwarf mosaic virus (MDMV)</i>	P1 protein (protease)	Intron-hairpin-RNA	Resistance	158

their biogenesis. For example, siRNAs are regulatory molecules of approximately 21–24 nucleotides in length, the product of double stranded (dsRNA) precursor processing, originated from transgenes, endogenous repeat sequences, viruses, or transposons, and act through post-transcriptional gene silencing or transcriptional gene silencing pathways. Several siRNAs have been identified to date based on the nature of the loci and biogenesis, such as natural antisense siRNA (natsiRNA), trans-acting siRNA (tasiRNA), and repeat associated siRNA (rasiRNA), and others. Recently, two distinct classes of virus-induced siRNAs (vsiRNAs) have been reported. This includes primary siRNAs directly processed by Dicer from initially triggered RNA as well as secondary siRNAs produced by RNA-dependent RNA polymerase (RdRP) enzyme.<sup>23</sup> The miRNA genes constitute ~1% of the total coding genes and form the largest class of regulatory molecules in flies, worms, and humans.<sup>24</sup> A number of small RNAs have been reported from different organisms and sources, indicating their diverse role in biological pathways.<sup>25</sup> The enzymatic machinery required for the generation of several classes of small RNAs are listed in Table 2.

### RNA Interference Pathway

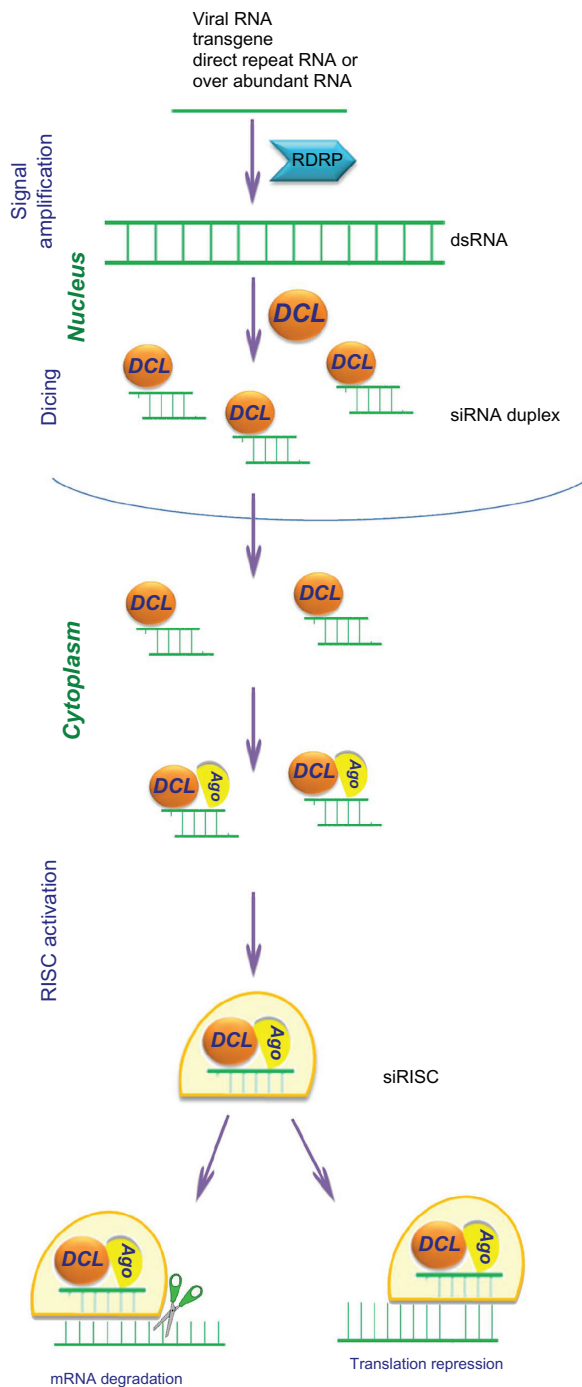
RNAi is a highly conserved pathway in eukaryotes in the network of interconnected defense responses that are activated during viral infection. In addition to being a fascinating biological process, it also provides a revolutionary technology that has guided many genes to be knocked down for functional analysis, nutritional quality improvement, and pest resistance.<sup>21,26,27</sup> The antiviral silencing pathway can be divided into the major steps described below (Fig. 1).

### Dicing

In this step of RNA silencing, RNase III type endonucleases (Dicer) act on its substrate dsRNA to generate siRNAs. Plants possess multiple Dicers; for instance, *Arabidopsis* has four Dicer-like (DCL) enzymes including DCL 1, 2, 3, and 4. DCL1 is responsible for miRNA generation, while DCL2, DCL3, and DCL4 are required for siRNA generation. Interestingly, a previous study found that DCL2, DCL3, and DCL4 in *Arabidopsis* process both replicating viral RNAs and RNAi-inducing hairpin RNAs (hpRNAs) into 22, 24, and 21 nucleotide siRNAs, respectively, and that loss of both

**Table 2.** Different classes of small RNAs, their roles, and enzymatic machinery involved in their biogenesis.

Small RNA	Size	Precursor	Enzymes required for biogenesis	Functions	Reference
siRNA (Small interfering RNA)	~21–24 nt	dsRNA processed from transgenes, endogenous repeat sequences, virus or transposons	DCL2/3/4/Ago2	Post-transcriptional gene silencing, viral defense	30
tasiRNA (Trans acting siRNA)	~21 nt	Small RNAs generated from endogenous transcript as template	DCL4/RDR6/Ago7	Direct the cleavage of protein-coding transcripts	159
rasRNA (repeat associated siRNA)	~24–26 nt	Products of dsRNA resulted during unchecked transcription event, usually retro-transposon loci and were found in mammalian germline cells	Dicer independent and function through piwi rather than Ago	Silencing of genomic repeats, including transposable elements	160
natsiRNA (natural antisense transcript siRNA)	21–24 nt	Are derived from endogenous overlapping <i>cis</i> -natural antisense transcripts (NAT), are induced by abiotic and biotic stresses or accumulate in specific developmental stages	DCL1/RDR6 PolIV in Arabidopsis	Regulation of stress-response genes	161
casRNA (chromatin associated siRNA)	24–25 nt	Produced from transposable elements, heterochromatic regions or other repetitive sequences	DCL3/RDR2/Ago4	Direct the DNA methylation on the genomic loci where they originate from and silence the residing transposable elements in <i>cis</i>	162
IsiRNA (Long siRNA)	30–40 nt	Induced in response to bacterial infection	DCL1, DCL4/AGO7	Induce decapping and 5' to 3' mRNA degradation of their targets	163
miRNA (microRNA)	21–24 nt	Derived from endogenous single stranded RNA precursor containing short, imperfectly paired stem of a much larger foldback transcript	DCL1/Ago1	Gene regulation, translational repression, heterochromatin production etc.	24
piRNA (Piwi interacting RNA)	25–30 nt	Derived mainly from repetitive elements, transposons and large piRNA clusters	Dicer independent/Ago3	Regulation of transposable elements in insects, mammals and zebrafish and are accumulated specifically in the germline	164
diRNA (DNA double-strand breaks (DSBs)-induced small RNAs)	~21 nt	Are produced from the sequences in the vicinity of DSB sites in Arabidopsis and in human cells	PI3 kinase ATR, RNA polymerase IV (Pol IV), Dicer-like proteins and AGO2	Functions as guide molecules directing chromatin modifications or the recruitment of protein complexes to DSB sites to facilitate repair	165
vsRNAs (virus derived siRNAs)	21–22 nt	ds viral RNA replication intermediates, local self-complementary ds regions of the viral genome and action of RDRs on viral RNA templates	DCL4, DCL2, AGO1, RDR6 and RDR2	Plant defense	23,166,167



**1. Signal amplification**

The single stranded RNA to be silenced is recognized by an enzyme RDRP which leads to formation of dsRNA. Accumulation of dsRNAs induces RNA silencing pathway and the signal is amplified by host or viral encoded RDRPs.

**2. Dicing**

The RNAi inducing dsRNA molecules are sequentially acted on by RNase III type endonucleases (Dicer like protein (DCL)) to generate duplex RNAs of size range 21–24 nts.

**3. RISC activation**

The single stranded mature small RNAs act as guide molecules to multi-protein complex called RISC. The major component of RISC is Ago protein. The helicase activity of Ago protein initiates dsRNA unwinding together with other required proteins of RISC.

**4. mRNA degradation**

siRISC activates the RNase activity of Ago protein which cleaves target complementary sequence and degrades the mRNA. RISC cleaves targeted mRNA in cases of perfect or near-perfect base pairing between mRNA and short guide RNA, whereas it mediates translation repression in cases of partial complementarity.

**Figure 1.** The basic steps of virus-induced or transgene-induced RNA silencing pathway. There are four steps of viral gene silencing as shown in figure.

*dcl2* and *dcl4* activities is required to negate RNAi.<sup>30</sup> Similarly, six DCLs have been identified in rice,<sup>31</sup> which are likely involved in the generation of a different class of small RNAs or may have redundant activities.

**RISC activation**

The multi-protein complex called RNA induced silencing complex (RISC) is comprised of Argonaute

(Ago) protein, small RNA, and several other proteins that mediate RNAi through sequence-specific complementarity. In plants, different Ago proteins are members of si and mi RISC. The functions of various members of Argonaute proteins in different species have been discussed previously in detail.<sup>32</sup> In *Arabidopsis*, Ago1 is mainly involved in miRNA-mediated gene silencing and tasiRNA,

Ago-4 generates rasi-RNAs that participate in RNA-directed DNA methylation, Ago-6 plays a role in DNA methylation and transcriptional gene silencing, while Ago-7 has been shown to generate tasi-RNA and long siRNAs.<sup>30</sup> Ago-1 and Ago-7 function to ensure the efficient clearance of viral RNAs, and Ago-7 seems to work as a surrogate slicer in the absence of Ago-1. Moreover, it is likely that Ago-1 is capable of targeting viral RNAs with more compact structures, whereas Ago-7 favors less structured RNA targets.<sup>23,33</sup> The role of different Ago proteins in antiviral silencing has been thoroughly addressed in recent reports.<sup>34–37</sup>

### mRNA degradation

The mature siRNA with Ago protein is incorporated into a multi-protein complex RISC which guides the cleavage or translational repression of the target mRNA or viral RNA by sequence-specific base-pairing.

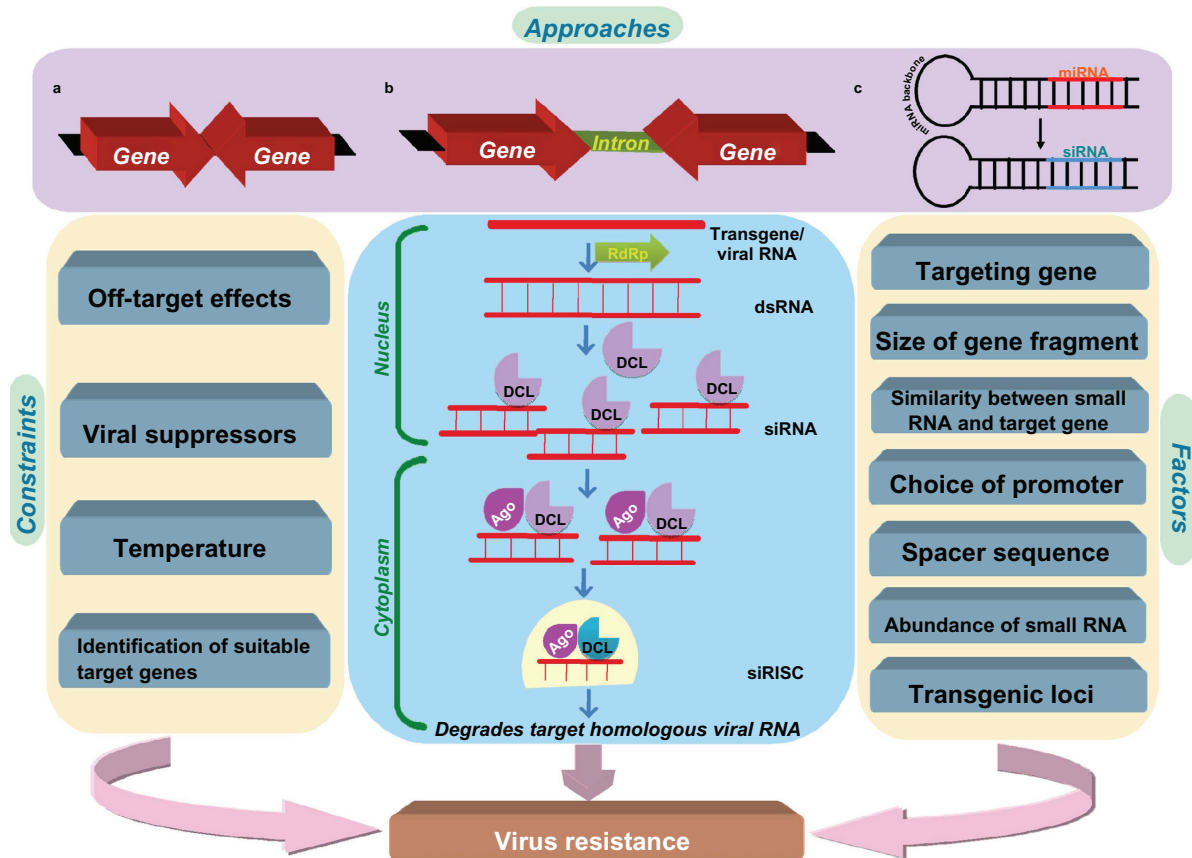
### Signal amplification

Single-stranded aberrant RNAs are used as templates by an RNA-dependent RNA polymerase (RdRP)

enzyme to synthesize dsRNA in a primer-dependent or primer-independent manner. The dsRNA is subsequently processed into secondary siRNAs by a Dicer or DCL nuclease. Recent studies have shown that the RNAi-mediated viral immunity in *Arabidopsis thaliana* requires host RDR1 or RDR6 to produce viral secondary siRNAs, which leads to viral RNA replication-triggered biogenesis of primary siRNAs.<sup>28</sup> Interestingly, it was found that RNA viruses also encode their own RdRP that synthesizes dsRNA replicative intermediates during viral RNA replication, which are sufficient to induce antiviral silencing.<sup>29</sup>

## Approaches for RNAi-Mediated Resistance

A variety of approaches have been attempted to express cognate dsRNAs to viral transcripts in order to initiate the process of viral gene silencing (Fig. 2). Initially, this was achieved by separately expressing sense and antisense genes of viral origin in plants and bringing them under a single genetic background by crossing. These studies also revealed that dsRNA is a



**Figure 2.** An overview of approaches, factors, and constraints of RNAi-based virus resistance development in plants.



potential elicitor of RNAi compared to the sense and antisense form of genes separately.<sup>10</sup> In plants, engineered inverted repeat (IR) transgenes derived from viruses were developed to express RNAs capable of duplex formation, thereby conferring resistance to the homologous invading viruses.<sup>38,39</sup> A comparison of the silencing ability mediated by the hairpin RNA and antisense RNA showed that the former accounts for 90%–100% increase in gene silencing compared to the latter.<sup>40</sup> The strategy of employing short-hairpin RNA (sh-RNA) vectors to achieve silencing of genes with viral and other endogenous origins has been widely explored.<sup>41</sup> During the past few years, advanced approaches of RNAi based on hairpin constructs and artificial microRNA (amiRNA) have been successfully employed in various hosts and viruses. Numerous efforts have refined the criteria of developing an effective antiviral RNAi construct. Approaches based on direct delivery of dsRNAs are other advanced antiviral strategies that have great potential in the future; however, more effort is needed to demonstrate its effectiveness in the field. This section describes various approaches of RNAi-inducing construct development that can effectively trigger and initiate RNAi and lead to high resistance frequencies in transgenic plants.<sup>42,43</sup>

### Sense/antisense RNA

The first transgene constructs used to induce RNAi were antisense and sense (co-suppression) constructs that were stably transformed into plants.<sup>44</sup> Approaches for expressing sense or antisense RNA in transgenic plants have been employed successfully against *Tomato golden mosaic virus* (TGMV),<sup>45</sup> *Tomato yellow leaf curl Sardinia virus* (TYLCSV),<sup>46</sup> and *Tomato yellow leaf curl virus* (TYLCV),<sup>47</sup> confirming that these methods can be harnessed for antiviral defense.<sup>48</sup> Transgenic *A. thaliana* plants were generated by introducing the full coding region of *Turnip mosaic virus* (TuMV) coat protein (*cp*) gene to silence its expression. The transgenic plants were completely resistant not only to the derivative TuMV isolate, but also to 17 different TuMV isolates collected worldwide.<sup>49</sup> Efforts were made to suppress the *Rice tungro bacilliform virus* (RTBV) by expressing DNA encoding ORF IV of RTBV, both in the sense and anti-sense orientations, resulting in the formation of RNAi-inducing dsRNA, where the transformed

rice plants showed tolerance to the virus disease.<sup>50</sup> The sense and antisense approach of RNAi has been attempted in many other crops as well, such as cotton, where *Cotton leaf curl disease* (CLCuD)-resistant cotton was developed using an antisense coat protein. Transgenic plants remained asymptomatic when screened for the virus resistance by inoculating with viruliferous whiteflies.<sup>51</sup> Although attaining virus resistance using a sense/antisense approach of RNAi has been quite successful, there are some instances in which this approach did not produce the expected results. RNA-mediated silencing of the rep-mRNA of the TYLCSV, on either the simultaneous expression of sense and antisense RNA or sense RNAs in multiple copies, could not be sustained. However, the high pressure of the viral inoculum on whitefly-mediated transmission of the virus overcame the resistance through RNA silencing.<sup>52</sup> Nevertheless, these plants often showed significant delays in symptom development, particularly at low inoculum dosage.

### Inverted repeat (IR) RNA

Inverted repeat (IR) constructs are composed of inversely repeated sequences of the target gene. Many studies have demonstrated that IR sequences of partial cDNA from a plant virus can silence the corresponding virus gene. Transgenic tobacco lines containing IR of *Cucumber mosaic virus* (CMV) cDNA were generated with a transgene capable of producing intramolecular dsRNA homologous to the 3' portion of the RNA3 genome. When the transgenic plants were challenge inoculated with CMV, three different types of plants were obtained, including susceptible, recovered plants, and asymptomatic resistant plants.<sup>39</sup> In another study, IR constructs encoding self-complementary dsRNA molecules of the *cp* gene sequences were transformed and expressed in *Nicotiana benthamiana* plants. Transgenic tobacco plants showed resistance to CMV.<sup>42</sup> Using a similar approach, transgenic sugarbeet plants were generated expressing IR of 400 base pairs (bp) from the *replicase* gene of the *Beet necrotic yellow vein virus* (BNYVV). Upon challenge inoculation with virus spreading vector species *Polymyxa betae*, the transgenic lines exhibited resistance, even under high inoculation pressure.<sup>53</sup> Many such reports of IR constructs targeting the *cp* gene have shown resistance, including *Soybean dwarf virus* (SbDV), *Cucumber green mottle mosaic virus*





(CGMMV), and TYLCV.<sup>54–56</sup> Transgenic lines resistant to *Papaya ringspot virus-W* (PRSV-W) indicated that the IR construct of *cp* gene had a high efficiency (100%) for inducing RNAi to protect the cantaloupe against PRSV-W infection.<sup>57</sup> The IR construct containing the conserved region of movement protein (*mp*) of *Grapevine Fan leaf virus* (GFLV) was transformed in *N. benthamiana*, in which T1 transgenic lines resulted in plants showing resistance, recovery, retarded infection, and susceptibility phenotypes.<sup>58</sup> In addition, the IR construct-based RNAi approach has been attempted in many crops against different viruses and has shown promise in some cases; however, high levels of resistance have not been observed.

### Hairpin RNA (hpRNA)

The hpRNA transgene construct also contains inversely repeated sequences of the target gene-like IR; however, the repeat sequences are separated by another sequence known as a spacer. It is designed in such a way that the RNA transcribed from the transgene hybridizes with itself to form a hairpin structure. This is comprised of a single-stranded loop, encoded by the spacer region, and a base-paired stem encoded by the IR sequences. The spacer region is needed to stabilize the transgene construct. *Agrobacterium*-mediated transient expression of short hairpin RNA was found to inhibit TMV in *N. tabacum* plants.<sup>59</sup> Furthermore, *N. benthamiana* plants infiltrated with cultures of *Agrobacterium* carrying a hairpin RNA construct derived from the 54-kDa region of *Pepper mild mottle virus* (PMMoV) showed resistance to subsequent infection by PMMoV. At an interval of 3 or more days between the agro-infiltration with PMMoV hairpin RNA and virus inoculation, plants were protected against virus infection, as indicated by the absence of viral RNA.<sup>60</sup> Similarly, in barley, *Barley yellow dwarf virus* (BYDV)-derived hairpin RNAs from a polymerase gene sequence conferred immunity to the virus in an effective manner.<sup>38</sup> Such an approach was followed in potato plants in which hairpin RNAs corresponding to a conserved region of the *cp* gene of PVY were used. The transgenic plants were found to possess siRNAs homologous to the *cp* gene of PVY. Transgenic lines producing siRNAs were highly resistant to three strains of PVY.<sup>61</sup> Yadav et al<sup>62</sup> designed the RNAi hairpin *cp* gene construct of *Cassava brown streak Uganda virus* (CBSUV)

and transformed cassava plants. That study showed the presence of siRNA, which provided 100% resistance to virus across replicated graft inoculation experiments. Recently, a hairpin RNAi construct was designed to target the gene for nonstructural Pns9 protein of *Rice gall dwarf virus* (RGDV). All progenies from transgenic plants showed strong and heritable resistance to RGDV infection and did not allow virus propagation.<sup>63</sup> Overall, the hairpin approach of RNAi has been one of the most successful approaches, underscoring its potential in achieving resistance against different viruses in different plant species.

### Intron hairpin RNA (ihp RNA)

The ihpRNA transgene is similar to the hairpin transgene except that the spacer region is an intron sequence, as it enhances the efficacy of silencing. ihpRNAs are thought to be more readily produced and remain stable through the use of a selectable marker, thus providing a convenient alternative to the use of noncoding spacers. Pooggin et al<sup>64</sup> demonstrated that ihpRNA targeting the intergenic region of the bidirectional promoter of *Mungbean Yellow Mosaic Virus-vigna* (MYMV-vig) resulted in resistance to this virus. This finding was important as it unequivocally demonstrated that RNAi is an effective strategy for combating DNA virus infections in plants. To induce RNAi against TYLCV, an ihp construct containing 726 nucleotides of the 3' end of the *C1* gene separated by castor bean catalase intron was developed. Transgenic tomato plants containing a single transgene copy showed immunity to TYLCV, even under extreme conditions of infection.<sup>65</sup> Intron hairpin RNA-mediated resistance was successfully achieved against *Plum pox virus* (PPV) using the constructs designed to produce a silencing effect against P1 and HC-Pro genes of the virus.<sup>66</sup> Tobacco plants transformed with chimeric coding and noncoding sequences from *Citrus tristeza virus* (CTV) using constructs designed to produce self-complementary transcripts from *cp*, *p20*, *p23*, and 3' untranslated region (UTR) were positive for siRNAs and resistant to the virus.<sup>67</sup> Similarly, to generate engineered resistance to TYLCSV, tomato plants were transformed with an ihpRNA construct containing a truncated replication-associated protein (*rep*) gene. The transgenic plants were agro-inoculated with an infectious strain of TYLCSV and were found to be fully resistant.<sup>68</sup>



Intermolecular ihpRNA targeting the partial TMV *mp* gene and the partial CMV *rep* gene was introduced in tobacco. Transgenic plants showed resistance against both the viruses and remained stable in T4 progeny.<sup>69</sup> In another report, ihpRNA transcripts corresponding to *cp*, *54 K*, or *24 K* genes of *Citrus psorosis virus* (CPsV) were generated. Transgenic sweet orange plants expressing ihpRNA containing the *cp* gene showed high level of virus resistance.<sup>70</sup> One of the most successful applications of the ihpRNA-based approach of RNAi was the development of a BGMV resistant common bean. This is the first virus resistant transgenic event based on an RNAi approach to be deregulated in Brazil. The designed ihpRNA was directed against replication initiator protein mRNA of the virus. The transgenic common bean plants exhibited resistance, even under high pressure of 300 viruliferous whiteflies, upon challenge inoculation.<sup>71</sup>

### Artificial miRNA (amiRNA)

A recent technological innovation has added to the use of RNAi for managing viral diseases of plants and is known as artificial microRNA (amiRNA)-mediated silencing of viral gene expression in plants. This is an evolving approach in RNA-based gene silencing that utilizes the intrinsic property of host gene regulation (ie, microRNA). This innovative approach deploys the manipulated host miRNA pathway to achieve virus resistance. The finding that the 21 nucleotides of endogenous miRNA can be altered or replaced with other sequences without disturbing its biogenesis and maturation have increased interest in using miRNA as a tool to silence transcripts of interest.<sup>72</sup> Using *A. thaliana* miRNA backbones, Alvarez et al<sup>73</sup> and Schwab et al<sup>74</sup> demonstrated the applicability of the amiRNA approach in silencing the endogenous transcript(s). To achieve virus resistance, pre-miR159a of *Arabidopsis* was modified to generate artificial pre-miRNAs159 (pre-amiRNAs159) containing sequences complementary to genomes of two plant viruses, TYMV and TuMV. Transgenic lines carrying 35S-pre-amiRNA159 showed specific resistance to either TYMV or TuMV, depending on the expression of the cognate amiRNA. Moreover, transgenic plants that expressed both amiRNAs were resistant to both viruses and the virus resistance trait was heritable through at least 3 generations.<sup>75</sup> Moreover, expression of amiRNA targeting the *2b* gene or coding

sequence shared by the *2a* and *2b* genes of CMV could efficiently inhibit the gene and conferred effective resistance to CMV infection.<sup>76,77</sup> This approach has better prospects, as expression of several amiRNAs targeting multiple viruses can be used to generate multiple virus resistance. When *N. tabacum* was transformed with amiRNA, representing the silencing suppressor HC-Pro of PVY and p25 of *Potato virus X*, transgenic plants showed a high level of resistance to both viruses.<sup>78</sup> This approach appears superior, particularly in the context where siRNA-mediated silencing is nullified due to the temperature effect,<sup>79,80</sup> as amiRNA-mediated resistance works even at a low temperature.<sup>75</sup>

### Direct delivery of dsRNAs

Direct delivery of dsRNA includes the introduction of either chemically synthesized potent siRNAs or crude extracts of bacterially expressed dsRNAs by a simple procedure of spraying them onto plant surfaces. Tenllado et al<sup>81</sup> showed the direct delivery of dsRNA in plants by spraying crude extracts of bacterially expressed dsRNAs of PMMoV and PPV. Similarly, Gan et al<sup>82</sup> demonstrated the inhibition of *Sugarcane Mosaic Virus* (SCMV) infection in maize plants by using the direct delivery approach of RNAi. Crude extracts of *Escherichia coli* HT115 containing large amounts of *cp* gene-specific dsRNA were applied to plants as a spray to show the effectiveness of dsRNA derived from the *cp* gene in controlling SCMV. Similarly, the *cp* gene (480-bp fragment) of PVY was used as a target to down-regulate PVY mRNA expression *in vitro*. A total of six siRNAs were designed and screened through a transient transfection assay and knockdown of CP-PVY mRNA was calculated in CHO-k cells. The study found that one *cp* gene-specific siRNA out of a total of six was found to be the most effective for knockdown of CP-PVY mRNA in transfected CHO-k cells.<sup>83</sup> This study is important because RNAi-based silencing is highly sequence-specific and is important for selecting such sequences while aiming for virus resistance development in crop plants. While the direct delivery of dsRNAs to attain virus resistance is comparatively safer and a promising approach, it has not been widely accepted due to the instability of dsRNA, non-heritability, and inconsistent results shown in different experiments.



## Virus induced gene silencing (VIGS)

Virus-induced gene silencing (VIGS) is an RNAi approach used primarily for characterizing the function of plant genes through gene transcript suppression and is increasingly used in transient loss-of-function assays.<sup>41</sup> VIGS is an siRNA-mediated silencing method that uses viral vectors carrying a fragment of a gene of interest to be silenced in order to generate long dsRNAs, which are then processed by host RNAi machinery to produce siRNAs to silence the target gene. Typically, 300–800 nucleotide fragments of target gene sequences are used, but sequences as short as 23–60 nucleotides can also be effective.<sup>84,85</sup> *Tobacco rattle virus* (TRV)-based vectors are the most widely used VIGS and have been used to silence genes in several Solanaceous crops.<sup>86</sup> Recently, a VIGS vector for gene silencing in rice was developed from RTBV. To modify the cloned RTBV DNA as a VIGS vector, a tissue-specific RTBV promoter was replaced by a constitutively expressed maize ubiquitin promoter.<sup>87</sup> *Bean pod mottle virus* (BPMV) and *Cabbage leafcurl geminivirus* (CbLCV) genomes were also used as VIGS vectors.<sup>88,89</sup> There are several examples that validate the VIGS approach for suppressing expression, and several signs indicate that multiple gene silencing by VIGS is possible. Simultaneous silencing of two or more genes by insertion of two gene sequences into the vector has been successful for TGMV-VIGS in *N. benthamiana*,<sup>90</sup> for the CbLCV system in *Arabidopsis*,<sup>91</sup> and for the DNAb system in tomato.<sup>92</sup> This approach of RNAi has been explored mainly for plant functional genomics. This method also has potential to be utilized as an analysis tool to achieve virus resistance. Although it is not an approach for developing RNAi-mediated virus resistance, it is an excellent tool for screening different genetic regions of a viral genome *in vivo* for their silencing efficacy in order to develop effective virus resistance.

## Challenges of Antiviral RNAi Approaches

Although RNAi is explored largely to achieve resistance against many plant viruses, it has some constraints that require attention and methodical planning for effective and efficient RNAi applications (Fig. 2).

## Application of next-generation sequencing (NGS) to identify suitable target genes of viruses

The key of an RNAi-based virus resistance approach is mainly dependent on careful target selection and a convenient delivery system. To date, screening of known genes and cDNA libraries are the main methods of verifying optimal target gene selection for RNAi, though the scope of selection is limited. With the advancement of next-generation sequencing (NGS) technologies, it would be interesting to look for new targets of RNAi that may be more efficient and effective in achieving virus resistance. Comprehensive transcriptomic studies may provide insight in identifying novel targets. In particular, it would be interesting to determine the relative population of siRNAs originated from different viral genes through NGS technologies. This in turn, will be important for understanding the efficacy of different genomic components of viruses in siRNA generation capacity. It will also help in deciphering whether one particular gene is able to produce the same amount of silencing or siRNA production in various hosts of a virus. Miozzi et al identified viral and host transcripts targeted by viral siRNAs from two grapevine-infecting viruses (*Grapevine fleck virus* and *Grapevine rupestris stem pitting-associated virus*) using NGS tools.<sup>128</sup> Recently, several developments in RNA sequencing methods have provided even more complete characterization of RNA transcripts.<sup>145</sup> The future of RNAi technology will be highly dependent on identifying the most efficient target genes that will be specific to a virus and host plant. For better insight on this topic, readers may refer to a review on the application of NGS tools in virology and viral/host transcriptomics.<sup>146</sup>

## Off-target effects

RNAi-mediated mRNA degradation was thought to be exquisitely specific, requiring near identity between the siRNA and the target mRNA. However, some evidence suggests that siRNA does not always target a specific gene, thus resulting in non-specific gene silencing in some cases. This non-specific gene silencing often appears to be caused by silencing of homologs to the targeted gene and/or other genes sharing partial sequence complementarity to the siRNA.<sup>123,124</sup> Such non-specific effects of siRNA



that lead to the degradation of mRNA of genes that are not the intended silencing targets lead to off-target silencing. Potential off-target silencing effects of RNAi in *Arabidopsis* and *N. benthamiana* have been reported.<sup>124</sup> Off-target effects have been widely observed from large-scale screens in animals as well.<sup>123,125</sup> A recent study demonstrated that yellowing symptoms in *N. tabacum* caused by CMV is due to silencing of host ChII (chlorophyll biosynthetic gene) by viral siRNAs. Y-satellite RNA (Y-Sat) of CMV shows complementary sequences with ChII mRNA and Y-sat-derived siRNAs in the virus-infected plant down-regulate the mRNA of ChII by targeting the complementary sequence. Interestingly, symptoms produced by Y-Sat infection were rescued by transforming tobacco with a silencing-resistant variant of the *CHLI* gene, suggesting that off-targeting by plant viruses could be detrimental in RNAi-mediated resistance development using viral target genes.<sup>126,127</sup> Host transcripts targeted by vsRNAs derived from *Grapevine fleck virus* and *Grapevine rupestris stem pitting-associated virus* were identified that included several proteins involved in ribosome biogenesis and in biotic and abiotic stress.<sup>128</sup> Similar observations in another study suggest that siRNAs derived from viral sequences can affect the resistance phenotype by interfering in the expression of host genes.<sup>129</sup> The off-targeting effects can be reduced by using short sequences of viruses. Here, amiRNA may be a better choice for developing resistance, as only 21 nucleotides of virus are used to develop RNAi constructs, which means that there is a relatively lower probability of developing homologous sequences to host genes. Furthermore, the evolving data on viral and host genomics will certainly assist in predicting the possibility of off-target effects while selecting a viral target gene. This is a well-explored topic in recent publications<sup>6,130,131</sup> and can be referred to for better insight.

### Viral suppressors of RNAi

Although the effective functioning of the RNAi mechanism in various host systems is to combat the virus infection, viruses are strong enough to be established in the host by suppressing the defense mechanism. In response to RNAi, viruses have evolved specific strategies for counteraction to the molecular mechanism of immune resistance of plants. Most viruses have

evolved suppressor proteins that help in virus establishment by suppressing different steps of RNAi.<sup>16–18</sup>

The first report on direct involvement of viral suppressor proteins in interfering with the RNAi mechanism was demonstrated with the HC-Pro protein of potyvirus.<sup>9,132,133</sup> Since then, most plant viruses that have been studied to date possess a VSR, including viruses with positive, negative, or dsRNA genomes as well as geminiviruses with a single-stranded circular DNA genome. In general, the VSR encoded by each virus often targets only one of the steps of RNAi. Nevertheless, CTV and geminiviruses encode multiple VSRs, each of which have a distinct mode of action.<sup>41,134</sup> Different VSRs are involved not only in suppressing siRNA-mediated viral defense, but regulating miRNA-mediated pathways of plant development.<sup>17,135</sup> Interestingly, the HC-Pro suppressor of PRSV showed strong synergism with heterologous viruses suggesting the cross-boundary role of VSRs in suppressing RNAi-based defenses.<sup>136,137</sup> VSRs are a major hurdle in attaining RNAi-mediated resistance, and thus researchers have attempted to silence suppressor genes of viruses using RNAi approaches. An amiRNA targeting P69 suppressor of TYMV and HC-Pro suppressor of TuMV was developed to attain effective resistance against these two viruses.<sup>75</sup> Similarly, the 2b suppressor of CMV and p25 of PVX were silenced to achieve resistance.<sup>76,78</sup> However, silencing of VSRs may not provide effective resistance at all times due to the reasons discussed by Duan et al.<sup>138</sup> Several reports describe the role of VSRs in suppressing the host defense by various means, and readers may refer to literature that has appeared in this area for a better understanding.<sup>23,28,139</sup>

### Temperature

Most biochemical reactions are sensitive to temperature, and RNAi is not an exception. RNAi-mediated plant defense was also found to be temperature dependent.<sup>79</sup> At low temperatures, both virus- and transgene-induced RNAi are inhibited, which leads to enhanced virus susceptibility as well as a dramatic reduction in the level of virus- or transgene-derived siRNAs. RNAi is also activated and the amount of siRNAs gradually increases with elevated temperature. Halveda et al showed *Cymbidium ring spot virus* (CymRSV)-mediated RNAi induced symptom severity, while increasing temperature elevated



virus-derived siRNA accumulation, resulting in less symptom development compared to low temperature. Furthermore, the ability of defective interfering RNA to protect plants from CymRSV was shown to be more efficient at high temperature with elevated virus-specific siRNAs.<sup>140</sup> In *Drosophila*, temperature-dependent gene silencing by RNAi has been reported,<sup>141</sup> in which the RNAi effect on *Drosophila* sex differentiation observed at 29 °C was strongly inhibited at 22 °C. The effect of temperature was also studied in sweet orange against CPsV by Velazquez et al,<sup>142</sup> who showed moderate to intense symptom development in plants kept at low temperature (26/18 °C), while plants kept at high temperature (32/26 °C) did not exhibit symptoms. They suggested that the increase in temperature enhances the RNAi response of citrus plants and decreases virus accumulation. The role of temperature in RNAi-induced viral gene silencing was further supported by the finding that a VSR of PRSV (HC-Pro) binds small RNAs in a temperature-dependent manner.<sup>143</sup> The temperature regulated activity of *Arabidopsis* DCL2 to produce vsRNAs further strengthens the concept of temperature-dependent antiviral response of RNAi.<sup>144</sup> Fortunately, miRNA-based approaches of viral gene silencing, such as amiRNAs, are temperature-independent and have shown promise when temperature plays a crucial role in RNAi-based virus resistance development.<sup>75</sup>

## Factors Affecting RNAi-Based Virus Resistance

RNAi in plants is a natural defense system against viruses, which is influenced by a number of host, viral, and environmental factors (Fig. 2). However, the influence of these factors is case-specific. For instance, in many studies, the observed virus resistance level was correlated to the abundance of virus-specific siRNAs in plants, while in other reports, this correlation was not observed. Other important factors include the integration locus of a transgene as well as inherent characteristics of the transcript, transgene localization, its intermediate processing, and interaction with various proteins of competing machineries, size of gene fragment, promoter, and spacer sequence. These factors are considered to be critical for determining the fate of hairpin transcripts and efficient triggering of RNAi. The factors affecting RNAi-based virus resistance are discussed in this section.

## Target gene

Viral suppression using RNAi depends greatly on the gene/sequences used for dsRNA generation and RNAi induction. Interestingly, the effects of potential target sequences in each of the coding genes in the *Rice stripe virus* (RSV) genome were analyzed using transgenic rice plants that expressed a set of IR constructs. Transgenic plants that harbored IR constructs specific for the gene *pC3* encoding nucleocapsid protein as well as for *pC4* encoding a viral movement protein were immune to infection by RSV. In contrast, the IR construct specific for the gene for *pC2*, which encodes a glycoprotein of unknown function, and for *p4*, which encodes a major non-structural protein of unknown function, did not result in resistance. This study indicates that not all target genes are equally effective in preventing virus infection through RNAi.<sup>93</sup> Similarly, the non-structural protein genes *Pns12* and *Pns4* of *Rice dwarf virus* (RDV) were used to deliver resistance to rice plants. Transgenic plants harboring *Pns12*-specific RNAi constructs were strongly resistant, while transgenic plants expressing *Pns4*-specific RNAi constructs were less resistant.<sup>94</sup> Hairpin RNAs of the *PI*, *HC-Pro*, *P3*, *CI*, *Vpg*, *Nla*, *NI*, and *cp* genes of PVY were used to transform *N. tabacum*, where the percentage of resistant plants obtained ranged from 33%–64% depending on the target gene.<sup>95</sup> Plants expressing amiRNA carrying *2a* and *2b* viral genes were found to be more resistant and effective against CMV compared to transgenic plants expressing amiRNA targeting the 3' UTR of the viral genome.<sup>77</sup> Another study showed that dsRNA derived from the upstream region (*cp1*) was more effective than dsRNA derived from a downstream region (*cp2*) in controlling SCMV.<sup>82</sup> Recently, only one of three hairpin RNAi constructs targeting the overlapping genes of *Cotton leaf curl multan virus* (CLCuMV) involved in virus replication and pathogenicity was able to prevent systemic movement of the virus.<sup>96</sup> Furthermore, transgenic sweet orange plants expressing the *cp* gene showed a high level of virus resistance, while transgenic plants expressing *54 K* and *24 K* genes showed partial or no resistance.<sup>70</sup> These studies strengthen the view that not all virus-derived sequences will deliver efficient silencing-mediated resistance to viruses. This is an important criterion to take into consideration for virus resistance development using RNAi.



## Size of gene fragment

The selection of gene fragment size plays a crucial role in RNAi-based viral gene silencing. Several studies have examined the minimum size of target sequences necessary for RNAi-mediated knockdown. Gene fragments ranging from 50 bp to 1000 bp were used to successfully silence genes. Earlier studies suggested that the shorter the fragment, the less effective silencing achieved, while very long fragments increase the chance of recombination. Hence, a fragment length of between 400 and 800 bp was considered to be a suitable size to maximize silencing efficiency.<sup>97</sup> Transformation of tobacco plants with constructs expressing either 640 or 490 nucleotides of PVX movement protein RNA rendered plants fully resistant to the virus. In contrast, four of eighteen plants expressing a 320-nucleotide sequence were sensitive to the virus, while all plants expressing a 140-nucleotide sequence were sensitive.<sup>98</sup> Similarly, 75% of the tested plants that had been transformed with an RNA 2-derived 1534-nucleotide IR construct showed extreme resistance to CMV, while plants transformed with a similar construct of a 490-nucleotide sequence showed only 30% resistance.<sup>42</sup> The influence of insert size on VIGS efficiency has received little experimental attention. In 2008, Liu and Page<sup>99</sup> showed that cDNAs longer than approximately 1500 bp gave little or no silencing. Here, it was suggested that viral replication is impaired when a large foreign sequence is inserted in its genome. This could also be due to the removal of large inserts by recombination, in which silencing insert may have been lost.<sup>99</sup> Thus, it may be necessary to use individual virus segments of fewer than 200 bp. While such shorter segments have been utilized to either demonstrate RNA interference or obtain resistant transgenic plants, using very short segments is fraught with the risk of the siRNA being less able to interact with the small target. This may be due to local secondary structure in the target RNA, which makes it ineffectual or mutation in the viral target, particularly at the 5' end of the siRNA and/or off-target effects of the siRNA.<sup>100,101</sup> Although there are no accurate indications regarding the size of the insert to be used for RNAi-mediated virus resistance, various reports suggest that this parameter is critical for successful application of the technology.

## Homology between small RNA and target gene

The backbone of RNAi-based virus resistance is the sequence similarity between siRNA and target gene. In fact, one study suggested that the degree of sequence similarity of siRNA with the target virus gene is related to the resistance or resistance-breaking phenomena. Homozygous tobacco plants containing a 597 nucleotide hairpin RNA construct of the PVY *rep* sequence were challenged with a variety of PVY strains. The transgene-carrying tobacco line was immune to five potato PVY strains with 88.3%–99.5% sequence similarity to the transgene. In turn, infection with more distant tomato and pepper PVY field strains (86%–87% sequence similarity) caused delayed symptom appearance in the transgenic tobacco.<sup>102</sup> Similarly, transgenically expressed sequences of the tospovirus *Tomato spotted wilt virus* (TSWV) provide protection against the homologous virus, but not against the related viruses *Groundnut ringspot virus* (GRSV) and *Tomato chlorotic spot virus* (TCSV), which share 78%–80% nucleotide sequence identity across their genomes.<sup>103</sup> Mubin et al<sup>96</sup> expressed a binary vector carrying the *rep* gene of the CLCuMV in *N. benthamiana*. The construct efficiently prevented *rep*-induced necrosis of inoculated tissues for the PVX vector expressing CLCuMV *rep*, but not the PVX vectors expressing the *rep* genes of the distantly related *African cassava mosaic virus* (ACMV) and CbLCuV, whose *rep* sequence shares 82% and 86% nucleotide sequence identity, respectively, to the *rep* gene of CLCuMV.

## Choice of promoter

Similar to any other transgenic trait, the choice of promoter is critical for RNAi-induced virus resistance. The most commonly used promoters are constitutive promoters, such as the 35S and maize ubiquitin promoters. Constitutive plant promoters confer high levels of transgene expression in dicot plants, but were not expressed in all cell types in cereal plants, suggesting that the constitutive promoter systems are not effective for silencing genes in certain tissues/organs.<sup>104</sup> To overcome the limitations of constitutive promoters, regulated promoters such as tissue or stage-specific promoters<sup>105,106</sup> and physically/chemically-inducible promoters<sup>107,108</sup> have been used. These promoters will induce RNAi only in a specific organ/tissue,



at a certain stage, or in response to physical or chemical agents. Wang et al<sup>109</sup> suggested that Pol III promoters are a potentially useful alternative to Pol II promoters for directing silencing constructs in plants. With the advancement of RNAi technology, use of specific promoters for efficient silencing of targeted genes will be key. Although numerous studies have been conducted to address this issue, additional studies are needed to identify specific promoters that are effective for silencing of viral genes through RNAi.

### Spacer sequence

Many hairpin RNAi constructs have a spacer sequence between an inverted repeat that allows for transgene stability. Sequences prepared from the *GUS* gene,<sup>110</sup> *GFP* gene,<sup>111</sup> and several introns<sup>40,112,113</sup> have been used as spacer sequences for the RNAi construct. Several reports have shown that enhanced gene silencing can be established by using an intron spacer rather than by the *GUS* or *GFP* spacers.<sup>40,112,114</sup> Splicing of the intron is considered to promote formation of a dsRNA structure of nascent transcripts with IR sequences.<sup>115</sup> The degree and frequency of RNAi-based silencing is dependent on the nucleotide sequence and size of spacer.<sup>116</sup> These reports emphasize that the size as well as the sequence of the spacer region is extremely important for RNAi-based virus resistance. However, extensive studies in this direction will provide a much clearer view in the future.

### Abundance of siRNA

Many reports have recently been published regarding the abundance of siRNA, but there is currently no consensus due to differences in results obtained by different research groups. In an initial report, resistance was correlated with the level of expression of siRNAs from viral-derived sequences. Plant lines that failed to produce siRNAs at a detectable level were susceptible to CMV infection, whereas plants producing siRNAs were resistant to CMV.<sup>39</sup> Correlation exists between expression of the AC1-homologous siRNAs and ACMV resistance of the transgenic cassava lines. Transgenic cassava lines with high levels of AC1-homologous siRNAs showed ACMV immunity.<sup>117</sup> Similar studies on *Watermelon mosaic virus* (WMV), PRSV-W, PVY, PVA, PLRV, TRV, and PMTV showed that resistance to the viruses correlated with levels of accumulated siRNAs.<sup>101,118</sup> In contrast,

Lopez et al found that accumulation of transgene-derived siRNAs is not sufficient for RNAi-mediated protection. Mexican lime plants transformed with the 3'-terminal 549 nucleotides of the CTV genome in sense, antisense, and intron-hairpin formats were analyzed for transgene-derived siRNA accumulation as well as for CTV resistance. Resistance was always associated with the presence of siRNAs; however, it was correlated with low accumulation of siRNAs in intron-hairpin lines.<sup>119</sup> In a study performed in *Tomato bushy stunt virus* (TBSV) and GFLV using transgenic *N. benthamiana* plants, it was found that effective RNAi does not necessarily lead to a detectable accumulation of siRNA.<sup>120</sup> Taken together, the presence of siRNAs is an important factor for RNAi-mediated virus resistance. However, the correlation between the level of siRNAs and degree of immunity will require further examination in a case-specific manner.

### Transgenic loci

Recent development of RNAi-based viral resistance suggests that different transgene integration loci resulted in clearly distinct cytoplasmic and nuclear RNAi activity. One locus was found to be necessary and sufficient for the development of a resistant phenotype and produced substantial amounts of siRNAs-CMV. A second locus, although transcribed, failed to produce detectable levels of siRNAs-CMV and did not confer resistance.<sup>120</sup> Han et al<sup>122</sup> demonstrated previously that silencing is linked with a higher transgene copy number and/or a particular transgene locus. There are only a few reports on the effect of the transgene locus in RNAi-based virus resistance. Nevertheless, the transgene locus is a critical factor in the expression or transcription of a transgene, which has a direct impact on dsRNA production and the subsequent RNAi-based silencing of viral genes.

### Future Prospects

Recent advances have created high expectations for the incorporation of RNA-mediated traits in crop improvement. Perhaps the most important applications will be in altering host-virus interactions, which will provide protection for plants from viral pathogens. Efficient methods for RNAi as a means of controlling plant viruses have been success-



fully demonstrated in different host-virus systems. New approaches such as amiRNA promise to bring more precision and predictability to the technology. In addition, amiRNA approaches of RNAi vector constructs using short sequences of target genes and endogenous miRNA backbone sequences offer great potential towards meeting the challenges imposed by temperature and off target effects. Furthermore, utilizing viral suppressor genes for RNA silencing may help in counteracting the RNAi suppression mechanism of viruses. VIGS has been explored mainly for plant functional genomics. Nevertheless, it has potential to be utilized as an analysis tool for achieving virus resistance. Different genetic regions of the viral genome can be screened for their silencing efficacy in order to develop effective virus resistance. Silencing of viral genes is influenced by the approach used, and thus a number of factors will need to be addressed in future studies. Currently, problems with off-target effects, non-target effects, impact of genetic mutations and polymorphisms, efficacy, stability, and validation limit the uses of RNAi for both scientific and commercial applications. Thus, the rapid discovery and an increasing knowledge of viral genomics, small RNAs, and RNAi will lead to continuous improvements in the biotechnological uses of RNAi.

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## Author Contributions

Conceived and designed the experiments: SA, SKM. Analyzed the data: SA, SKM. Wrote the first draft of the manuscript: SA, SKM. Contributed to the writing of the manuscript: SA, SKM. Agree with manuscript results and conclusions: SA, SKM. Jointly developed the structure and arguments for the paper: SA, SKM. Made critical revisions and approved final version: SA, SKM. All authors reviewed and approved of the final manuscript.

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