

# Chapter 2

## Caveat of RNAi in Plants: The Off-Target Effect

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### Abstract

RNA interference (RNAi), mediated by short interfering RNAs (siRNAs), is one of the widely used functional genomics method for suppressing the gene expression in plants. Initially, gene silencing by RNAi mechanism was believed to be specific requiring sequence homology between siRNA and target mRNA. However, several recent reports have showed that non-specific effects often referred as off-target gene silencing can occur during RNAi. This unintended gene silencing can lead to false conclusions in RNAi experiments that are aimed to study the functional role of a particular target gene in plants. This especially is a major problem in large-scale RNAi-based screens aiming for gene discovery. Hence, understanding the off-target effects is crucial for minimizing such effects to better conclude gene function analyzed by RNAi. We discuss here potential problems of off-target gene silencing and focus on possibilities that favor this effect during post-transcriptional gene silencing. Suggestions to overcome the off-target effects during RNAi studies are also presented. We believe that information available in present-day plant science literature about specificity of siRNA actions is inadequate. In-depth systematic studies to understand their molecular basis are necessary to enable improved design of more specific RNAi vectors. In the meantime, gene function and phenotype results from present-day RNAi studies need to be interpreted with caution.

**Key words:** Off-target prediction, plant functional genomics, RNA silencing, siRNA scan, unintended silencing.

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### 1. Introduction

RNA interference (RNAi) technology has made pan-genomic functional gene analysis a reality and it is a powerful strategy for gene discovery and validation (1). RNAi is dominant, so the gene-silenced phenotypes can be observed in the T1 generation itself. RNAi often leads to partial knockdown of gene transcripts, thus providing a range of phenotypes that may differ in severity. RNAi facilitates the study of essential genes whose complete

inactivation would lead to lethality or extremely severe pleiotropic phenotypes. RNAi can be quickly and easily used in a wide range of genotypes or even species, whereas identification of gene mutations is limited to certain plant species which has the mutant resources. Expression of RNAi constructs can be controlled in a tissue-specific or time-dependent manner (2). There are several examples where loss of gene function results in improved plant performance (3–6). Thus, recently RNAi has been effectively used in transgenic crop development for commercial uses (3, 4, 6). One of the characteristics of RNAi is its ability to silence genes in a sequence-specific manner. However, recent evidences suggest that small interfering RNA (siRNA; RNAi pathway intermediate) does not always target a specific gene, thus resulting in non-specific gene silencing (7, 8). Non-specificity can occur when partial sequence homology allows siRNA to degrade mRNA for genes that are not the intended silencing targets. Several other deviations that can possibly occur at different steps during post-transcriptional gene silencing (PTGS) pathway can also favor such non-specific effect (7, 9–12). Such non-specific effect of siRNA to degrade mRNA for genes that are not the intended silencing targets is referred as off-target silencing.

Recently our group has reported potential possibilities of off-target silencing effects during post transcriptional gene silencing (PTGS) in *Arabidopsis* and *Nicotiana benthamiana* (8). Off-target effects have also been widely observed from large-scale screens in animals (7, 9). RNAi is a rapidly growing field with considerable interest to the plant science community for potential genomics research and agriculture applications. Hence, the potential deleterious off-target silencing needs to be eliminated. In this review, we attempt to provide detailed information about the nature of off-target silencing and also propose ways to overcome this effect.

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## 2. RNAi Mechanism and dsRNA Delivery into Plants

Long double-stranded RNA (dsRNA) can be used to silence the expression of target genes. Upon introduction, the dsRNAs enter a cellular pathway that provokes PTGS. The dsRNAs get processed into 21–23 nucleotide siRNAs by an RNase III-like enzyme called Dicer. In plants, Dicer-like 4 (DCL4) is predominantly involved in this step (13). Then the siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing complex (RISC). The siRNA strands subsequently guide the RISCs to complementary mRNA molecules, where they cleave and destroy the cognate mRNA. Cleavage of

cognate mRNA takes place near the middle of the region bound by the siRNA strand. These steps are elaborately reviewed elsewhere (1, 14, 15).

RNAi in plants can be achieved by expressing hairpin RNA (hpRNA) that folds back to create a dsRNA. These hpRNAs are potent inducers of PTGS and give rise to siRNAs derived from the dsRNA. Transforming plants with dsRNA-expressing vectors for the selected gene by *Agrobacterium*-mediated transformation or particle bombardment, infecting plants with viral vectors that can express dsRNA (virus-induced gene silencing), infiltrating *Agrobacterium* cultures harboring hpRNAi construct for transient gene silencing are commonly used methods for gene silencing in plants (8, 16–18). Most of these methods require vector construction and plant transformation. Direct siRNA delivery into plants is another method proposed for large-scale RNAi screens as in animal system. This method has been demonstrated using laser-induced stress wave in plant cell cultures (19). Apart from these, artificial microRNA (amiRNA)-based vectors have been recently shown to be effective for gene silencing (20). These vectors upon delivery into plant cells provoke the PTGS and are expected to silence genes of interest.

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### 3. General Adverse Effects of Off-target Gene Silencing

RNAi has been widely used as a reverse genetics tool for gene function characterization in plants. Our earlier study (8) computationally predicted that about 50–70% of gene transcripts in *Arabidopsis* plants have potential off-targets when used as silencing trigger for PTGS and this could obscure experimental results. Up to 50% of the predicted off-target genes tested in plants were actually silenced when tested experimentally (8). Apart from other studies (9, 11, 20), our group has also demonstrated that such off-target silencing lead to difficulties in identifying exact functional role of target genes by using stable RNAi or VIGS approaches (8).

Another use of RNAi or VIGS is its application in large-scale forward genetic screens for gene discovery and functional analyses. Impact of off-target silencing during large-scale screening in animals has been demonstrated (7, 9). We hypothesize that potential for off-target silencing also exists in high-throughput screens in plants (e.g., AGRIKOLA – *Arabidopsis* genomic RNAi knockout line analysis, <http://www.agrikola.org/index.php?o=/agrikola/main> or <http://www.chromdb.org/>) and is not yet reported simply because of lack of systematic study.

Yet another important application of RNAi is in the area of crop improvement. Today, researchers are engineering a variety

of crops to produce siRNAs that will silence essential genes in insect pests, nematodes, and pathogens, an approach called host-delivered RNAi (HD-RNAi) (3, 4, 21). Plant genes are known to share at least partial sequence similarity with animal genes. Specific domains of certain genes are conserved across different organisms. If off-targeting can unexpectedly silence genes in plant or pest, such unintended effects will raise concern not only about plant phenotypic pleiotropy but also in the organisms and the subsequent environmental consequences. In theory, vertical gene flow of an RNAi-mediated pollen lethality phenotype to native plants could alter fitness and biodiversity (3) and off-target silencing will only boost such negative effects. Hence this could be a potential biosafety issue for RNAi transgenic plants developed for crop improvement. Off-target silencing also poses threat to appli-

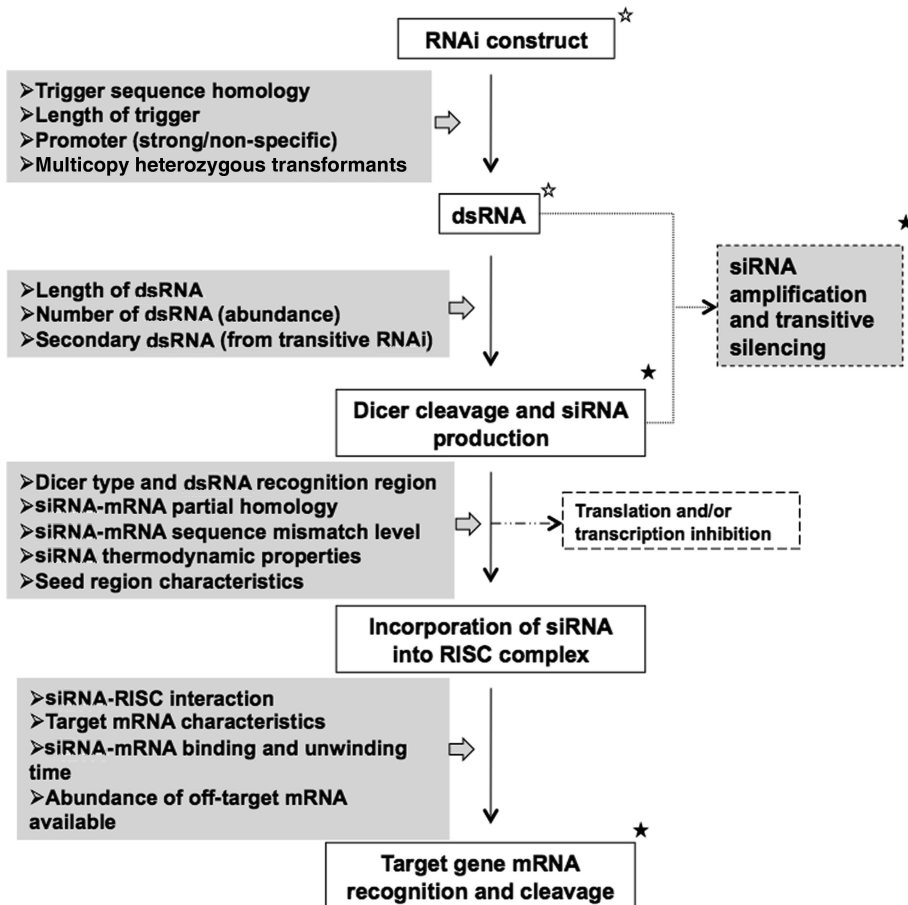


Fig. 2.1. Possible sources of off-target effects during RNAi in plants. The flow diagram shows RNA interference (RNAi) in transgenic plants with hpRNA vector along with potential possibilities of off-target induction in each step. ☆ Steps that can be manipulated to increase specificity of silencing. ★ Steps that cannot be manipulated at this time due to lack of information regarding mechanism.

cation of RNAi in developing several food crops with improved nutritional value. Especially, off-target effects if not controlled can severely compromise biosafety in RNAi plant food consumers, namely human and livestock. We discuss below the steps of PTGS pathway in plants that could potentially contribute to off-target gene silencing (*see Fig. 2.1*).

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## **4. Steps in PTGS and Their Relation to Off-target Activity**

### **4.1. RNAi Vector, Silencing Trigger, and dsRNA**

To develop genetically stable RNAi plants, binary vectors (called hpRNAi vectors) with gene fragments cloned in invert orientation separated by a short intron are widely used (8, 18, 22). The gene fragment used for producing dsRNA to silence the intended target mRNA is called as the “trigger.” Trigger sequence in RNAi vector plays important role in deciding specificity of gene silencing. Normally 250–350-bp fragment of gene is optimum as a trigger for hpRNAi vectors, while smaller lengths are also used in other methods, namely direct siRNA delivery and amiRNA vectors (8, 17–20, 22). First, length of dsRNA correlates with both silencing efficiency (23) and off-target silencing (11). While long hairpins are more likely to generate a diverse set of optimally effective siRNAs, they also have an increased potential to produce siRNAs with off-target effects (24). Essentially the chance of off-target increase with greater length of the initial dsRNA sequence has been demonstrated (11, 20). Second, number of dsRNA also has correlation with off-target silencing. Higher dsRNA than required threshold is known to favor off-target effects (7, 22, 25). Sometimes RNA-dependent RNA polymerase (RDR)-mediated amplification using small RNA or piece of dsRNA produces secondary dsRNA (26, 27). This can considerably influence the dsRNA number in cell. Apart from this, in the genetically stable RNAi plants, position of RNAi construct integration in the plant genome and copy number are factors deciding extent of its transcript expression (28, 29), thereby influencing the number of dsRNA production. To a certain extent, copy number and position of RNAi vector are influenced by the method used for plant transformation for delivering RNAi vectors. Third, use of strong promoters in RNAi vectors to drive hpRNA production could favor a completely different type of off-target effect owing to the inhibition of natural miRNA or siRNA regulation through saturation of the pathways with exogenous or transgene siRNAs (30). Fourth and most important aspect influencing off-target silencing is the nature of trigger sequence used in RNAi vector. Off-target silencing is mainly influenced by trigger sequence homology with mRNA. We have shown that

21-nucleotide stretch of 100% identity between the silencing trigger sequence and endogenous target gene sequences is not absolutely required to provoke gene silencing (8, 31). Computational tools also predicted the influence of trigger sequence used in hpRNAi or miRNA vector construction or siRNA design in off-target gene silencing. Apart from these, the silencing trigger sequence in hpRNA vector selected from the 3' UTR region of a gene is known to favor off-target gene silencing compared to a trigger from the 5' region (11).

#### **4.2. Dicer Cleavage and siRNA Production**

In RNAi *Arabidopsis* plants, dsRNA is predominantly processed by DCL4 to produce specific siRNA. However, other dicers, namely DCL2 and DCL3, can also be involved at this step and produce less specific siRNA, leading to unpredictable gene silencing (30). DCL action eventually decides length of siRNA, its diversity, and quantity (10). The dsRNA length and overhang features also influence dicer cleavage. For example, to efficiently process a short dsRNA, certain features like short overhanging 3' nucleotides on the dsRNA are beneficial and increase specificity. Further, excess unprocessed short dsRNAs can bind to mRNA without cleaving the molecule, preventing translation of the mRNA to protein. Such short dsRNAs can also bind to DNA, inhibiting the transcription of DNA to mRNA (24). The molecular mechanism behind these two actions is not yet completely understood and not reported in plants.

#### **4.3. siRNA Characteristics and Transitive Silencing**

siRNAs are the ultimate determinants of specificity of silencing. First and foremost, difficulty in maintaining targeted gene silencing is the limited sequence specificity of siRNAs. As few as 14 nucleotides (or even less) of sequence complementarity between siRNA and mRNA can lead to the inhibition of gene expression (8, 9, 11, 31, 32). Limited sequence specificity can potentially favor chances of siRNA sharing homology with off-target mRNA (30). A second specificity problem can occur via "transitive silencing," whereby RNAi against a gene-specific sequence spreads into neighboring sequences (26). In this process, the primary siRNAs bind to complementary transcripts and generate new dsRNAs via a 5' to 3' extension carried out by RDR. These newly synthesized dsRNAs can be processed into less specific secondary siRNAs, leading to silencing of any complementary mRNA. This process is called transitive RNAi and can affect the specificity of silencing. Transitive RNAi has been documented in many studies as a cause of non-specific silencing (23, 26, 27, 33). Further, this process can essentially amplify siRNAs, leading to off-target silencing. Such silencing effects can in some cases be transmitted from cell to cell and even over long distances throughout the plant, leading to a potential loss in control over the intended silencing signal (26, 30). An already non-specific source of siRNA or dsRNA

under these circumstances can further increase off-target effects and complicate understanding of target gene function from such an RNAi study. Hence, it is important to titrate the level of siRNA to lowest effective level to considerably reduce off-target silencing (7).

Apart from influencing PTGS, the off-target effects at siRNA level can also occur by other mechanisms. Excessive siRNA may function as miRNA and suppress translation without affecting transcript levels (12). Further, any siRNAs with only partial homology match with target mRNA can only loosely bind, leading to hindrance for ribosome access. This can also lead to prevention of protein synthesis by inhibiting translation without RNA silencing (34). Two other types of off-target effects include siRNA cross-hybridizing with undesired mRNA causing degradation and the binding of siRNA to protein (like transcription factors or other transcriptional regulatory enzymes) as an aptamer affecting transcription (34).

In addition, silencing potency of a perfectly matched siRNA is largely determined by the thermodynamic property of its primary sequence (20, 35, 36). Physical and chemical properties of siRNA are important to maintain specificity in gene silencing. GC content and stability of siRNA also play role in silencing and off-target activity (10). Off-target transcript silencing is widespread and mediated largely by limited target sequence complementarity to the seed region of the siRNA guide strand (32, 36, 37). A distinct hexamer sequence of the siRNA guide strand decides the pairing with complementary sequences in the off-targeted mRNA. This hexamer region between 2 and 7 nucleotides is called seed region. The siRNAs that has seed regions with high complement frequency with off-target mRNA can increase chances of off-target silencing. Modifications within siRNA seed region have been shown to reduce both the number of off-target transcripts (by 66%) and the magnitude of their regulation, without significantly affecting silencing of the intended targets (32). Seed region influence on off-target silencing is not widely reported in plants but has the potential to occur. When this phenomenon was investigated in more detail in mammalian cells, a strong correlation between off-targets and short stretches of complementarity to the siRNA in the 3' UTRs of the affected transcripts was found (37). Certain position-specific, sequence-independent chemical modifications in siRNA has also been shown to reduce off-target effects in animals (32).

#### **4.4. RISC Complex and mRNA Target Search Activity**

Generally, the antisense siRNA (guide strand) directs RISC to complementary mRNA, while the second (sense) strand is degraded. Off-target effects can be caused by insertion of the sense siRNA strand into the RISC complex instead of the antisense strand (7, 9, 35). During RNAi the active RISC complex



with siRNA samples target sites in mRNA non-specifically until the correct one is found. Once the correct target RNA is found, conformational changes occur that enhance RISC catalytic efficiency, leading to slicer activity (32, 35). However, binding of imperfectly matched targets can contribute to other forms of off-target effects, namely inhibition of translation (no slicing). Modifications to siRNAs that weaken or disrupt RISC–mRNA interaction in the seed region have been shown to reduce off-target silencing in human (32). Within the short sequence of siRNA, six base pairs (seed) play an important role in the binding of target mRNA to RISC. Studies by Jackson and colleagues have also shown that this seed region of siRNA shows the highest correlation with off-target effects (32).

#### **4.5. Target Gene mRNA Characteristics and Sequence Homology Required for Silencing**

Apart from siRNA–mRNA homology requirements, off-target silencing also depends on characteristics of target mRNA. This includes sequence context surrounding complementary regions, position of the complementary region in mRNA, and copy number of the complementary region (10). Studies suggest that each target sequence possesses an inherent degree of susceptibility to dsRNA-mediated degradation (28). Highly expressed genes are more amenable for unregulated silencing (28) as the frequency of activated RISC accessing their mRNA will be high. Yet another aspect that compromises the siRNA–mRNA specificity is nucleotide base pair mismatch, although plants can tolerate small number of mismatches compared to animals (10, 11, 24, 31). According to Warthmann and colleagues (20), any transcript other than the specified target, which matches the 21-mer with five mismatches, is considered an off-target (20). The efficiency of off-targeting due to mismatch depends on the length of mismatching nucleotides and their position on siRNA (11). Although only few of above-described steps in PTGS can be controlled by researchers to increase specificity of silencing during RNAi, future research can potentially facilitate control of other steps.

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### **5. Confirming the Specificity of Target Gene Silencing in RNAi Experiments**

Present RNAi studies in plants are likely to have unintended gene silencing effect (3, 8, 20). Hence ascertaining the specificity of silencing at the end of each RNAi experiment before interpreting results is important. Following are some useful tips: (a) Confirm reduction of target mRNA and its protein expression (without affecting related gene expression); (b) confirm specificity of the



observed phenotype; (c) perform gene expression profiling (e.g., microarray) to assay the expression pattern of non-target genes. Jackson and colleagues (7) have shown that expression profiling in conjunction with gene silencing by RNAi will provide an effective means to identify and characterize specific target gene function. (d) Rescue the RNAi effect by expressing an siRNA-resistant copy of target gene. Since most codons in the mRNA of the target gene can be altered at the third nucleotide position without changing the encoded amino acid, the RNAi-resistant copy of native mRNA can be designed to escape silencing (38). This should complement RNAi-induced effects and revert back to wild-type like plant. (e) Confirm that the same phenotype or change in specific metabolic process is produced when other regions of same gene are used as silencing trigger. (f) Examine multiple independent RNAi plants to check for a reproducible phenotype. (g) Compare the results with plant transformed with RNAi vector harboring non-plant gene (e.g., *GFP*). Monitor changes due to plant immune response (while using VIGS). (h) When silencing a gene family member, make sure that other family members are not silenced and vice-versa.

While performing these confirmatory experiments to ascertain the specificity of gene silencing, certain inherent limitations described below need to be kept in mind. (i) Certain genes are known to be induced upon perception of RNA silencing. Transcript reduction is not always directly correlated with phenotype or protein reduction. (ii) Silencing of certain genes may not show a visible phenotype. (iii) The full extent of the contribution of off-target gene regulation to phenotypic induction is not known. (iv) Similar to miRNAs, the effect of siRNAs on off-target protein regulation might be even greater than the effect on off-target transcript silencing. (v) Small changes in the expression levels of some proteins, such as transcription factors, might translate to large effects on phenotype. Until proteomic and metabolomic analyses of siRNA experiments are performed, we will not know the full consequences of siRNA off-target activity. (iv) Many endogenous genes are regulated by natural RNAi pathways, so perturbations in these processes can lead to pleiotropic effects (30).

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## 6. Remedy for Off-target Silencing

(a) Choose highly specific trigger sequence for RNAi vector. This can be done by considering several siRNA characteristics apart from sequence similarity alone. Minimize homology of trigger sequence in RNAi construct to non-target mRNA. If off-targets are predicted using computational approach, select the

trigger sequence which can avoid perfect siRNA stretches of less than 11 bp for predicted off-target gene. This should prevent off-target effects while maintaining adequate complementarity for target gene sequence. Computational off-target prediction tools are described in **Section 7**. (b) Wherever possible, use RNAi vectors with tissue-specific and inducible promoters to minimize off-target silencing. Although silencing might spread to other tissues, the benefit of specific promoters to increase specificity of gene silencing has been demonstrated (2, 39). (c) Maintain low-effective number of dsRNA and siRNA. This may be achieved by having appropriate promoters for RNAi plant generation and by analyzing single-copy homozygous transgenic plants. (d) Analyze many independent biological replicates and account for possible variations. (e) Since structurally different, RNAi-resistant, mRNA-producing construct that has same function with native gene can be artificially synthesized, certain off-target gene effects can be complemented by stable transgene development with RNAi-resistant, off-target gene construct. This method can be applied in commercially important RNAi transgenic plants to nullify off-target effect that occurred due to one or two genes.

Apart from these, some of following aspects could be considered when the adequate information and technology will become available in the future to control off-target silencing. (f) Minimize secondary siRNA production and transitive gene silencing. (g) Titrate the dsRNA and siRNA population to optimum numbers. (h) Minimize incorporation of the siRNA sense strand into RISC.

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## **7. Predicting Off-target Gene Silencing Using Computational Methods**

Computational prediction tools basically use sequence similarity to identify effective trigger sequence in RNAi vector. They are less expensive to implement and permit the extension of real parameters into wider ranges for fully observing the trends and effects upon RNAi specificity (11). BLAST has been the most commonly used tool to study homology between sequences. This can be utilized to search for sequences similar to the proposed siRNA in the desired organism. However, because of the short length of siRNAs, using BLAST requires attention to details and potentially relevant alignments can be easily missed. Hence, siRNA design rules were devised to check for sequence similarity with other genes, as well as to prevent the use of sequences that repeat or contain common binding sites, and look at the effect of the positioning of the siRNA on the target sequence. These design rules

are usually incorporated into algorithms to search for siRNAs that have matches for all gene sequences in addition to the targeted gene (8, 11).

Our group has developed a publicly available Web-based computational tool called siRNA Scan (<http://bioinfo2.noble.org/RNAiScan.htm>) to identify potential off-targets during PTGS in plants (8). The input parameters of the tool for analysis of siRNAs and off-targets can be adjusted by users. This siRNA scan tool is useful to design better constructs for PTGS by minimizing off-target gene silencing in plants. This tool includes the option of searching potential off-targets with complete sequence identity or reverse complementary identity of 18–29 nt to the trigger sequence, as well as allowing a few mismatches to the potential siRNAs. For designing amiRNA, a program called WMD2 (<http://wmd2.weigelworld.org>) is being used (20). Thermodynamics of the trigger sequence interaction with target mRNA can be analyzed using the program RNAup (36). Given the small degree of similarity implicated in off-target gene regulation, it may be difficult to select an siRNA sequence that will be absolutely specific for the target of interest merely based on sequence homology and few other above-described siRNA characteristics. Hence, better methods should be researched. New methods for creating RNAi vectors without non-specific effects need to look at interactions in the siRNA:RISC complex and how modifications of the siRNA can affect RISC. The computational tools will have full-fledged application once the nucleotide sequence information for vast majority of crop species becomes available.

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## 8. Future Perspectives

Plants adopt silencing strategy via miRNA pathway to control genes involved in organelle development and response against environmental stress through miRNA (13, 24, 35). The endogenous native expression of miRNAs in animals and plants does not create adverse effects but perform the specifically programmed role of these miRNAs in gene regulation. How does miRNAs maintain specificity in silencing? What are the mechanisms that regulate these naturally produced small RNAs? Future plant science research should be aimed to fully understand these questions. This could potentially reveal many ways to manipulate RNAi pathway to minimize off-target silencing. Further, a greater understanding of overall RNAi mechanisms specific to plants will allow for more possibilities in reducing off-target effects. Once the specificity of the siRNA is guaranteed, the objectives to use RNAi in studying gene function and other uses can be comfortably widened.

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## References

1. Waterhouse, P. M. and Helliwell, C. A. (2003) Exploring plant genomes by RNA-induced gene silencing. *Nat. Rev. Genet.* **4**, 29–38.
2. Shuai, C., Daniel, H., Uwe, S., and Fredrik, B. (2003) Temporal and spatial control of gene silencing in transgenic plants by inducible expression of double-stranded RNA. *Plant J.* **36**, 731–740.
3. Auer, C. and Frederick, R. (2009) Crop improvement using small RNAs: applications and predictive ecological risk assessments. *Trends Biotechnol.* **27**, 644–651.
4. Gordon, K. H. J. and Waterhouse, P. M. (2007) RNAi for insect-proof plants. *Nat. Biotechnol.* **25**, 1231–1232.
5. Niu, Q.-W., Lin, S.-S., Reyes, J. L., Chen, K.-C., Wu, H.-W., Yeh, S.-D., and Chua, N.-H. (2006) Expression of artificial microRNAs in transgenic *Arabidopsis thaliana* confers virus resistance. *Nat. Biotechnol.* **24**, 1420–1428.
6. Tang, G. and Galili, G. (2004) Using RNAi to improve plant nutritional value: From mechanism to application. *Trends Biotechnol.* **22**, 463–469.
7. Jackson, A. L., Bartz, S. R., Schelter, J., Kobayashi, S. V., Burchard, J., Mao, M., Li, B., Cavet, G., and Linsley, P. S. (2003) Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol.* **21**, 635–637.
8. Xu, P., Zhang, Y., Kang, L., Roossinck, M. J., and Mysore, K. S. (2006) Computational estimation and experimental verification of off-target silencing during posttranscriptional gene silencing in plants. *Plant Physiol.* **142**, 429–440.
9. Jackson, A. L. and Linsley, P. S. (2004) Noise amidst the silence: off-target effects of siRNAs? *Trends Genet.* **20**, 521–524.
10. Lin, X., Ruan, X., Anderson, M. G., McDowell, J. A., Kroeger, P. E., Fesik, S. W., and Shen, Y. (2005) siRNA-mediated off-target gene silencing triggered by a 7 nt complementation. *Nucl. Acids Res.* **33**, 4527–4535.
11. Qiu, S., Adema, C. M., and Lane, T. (2005) A computational study of off-target effects of RNA interference. *Nucl. Acids Res.* **33**, 1834–1847.
12. Saxena, S., Jónsson, Z., and Dutta, A. (2003) Small RNAs with imperfect match to endogenous mRNA repress translation. *J. Biol. Chem.* **278**, 44312–44319.
13. Dunoyer, P., Himber, C., and Voinnet, O. (2005) DICER-LIKE 4 is required for RNA interference and produces the 21-nucleotide small interfering RNA component of the plant cell-to-cell silencing signal. *Nat. Genet.* **37**, 1356–1360.
14. Agrawal, N., Dasaradhi, P. V. N., Mohammed, A., Malhotra, P., Bhatnagar, R. K., and Mukherjee, S. K. (2003) RNA interference: biology, mechanism, and applications. *Microbiol. Mol. Biol. Rev.* **67**, 657–685.
15. Meister, G. and Tuschl, T. (2004) Mechanisms of gene silencing by double-stranded RNA. *Nature* **431**, 343–349.
16. Bhaskar, P. B., Venkateshwaran, M., Wu, L., Ane, J.-M., and Jiang, J. (2009) *Agrobacterium*-mediated transient gene expression and silencing: a rapid tool for functional gene assay in potato. *PLoS ONE* **4**, e5812.
17. Higuchi, M., Yoshizumi, T., Kuriyama, T., Hara, H., Akagi, C., Shimada, H., and Matsui, M. (2009) Simple construction of plant RNAi vectors using long oligonucleotides. *J. Plant Res.* **122**, 477–482.
18. Senthil-Kumar, M., Hema, R., Suryachandra, T. R., Ramegowda, H. V., Gopalakrishna, R., Rama, N., Udayakumar, M., and Mysore, K. S. (2010) Functional characterization of three water deficit stress-induced genes in tobacco and *Arabidopsis*: an approach based on gene down regulation. *Plant Physiol. Biochem.* **48**, 35–44.

19. Tang, W., Weidner, D. A., Hu, B. Y., Newton, R. J., and Hu, X.-H. (2006) Efficient delivery of small interfering RNA to plant cells by a nanosecond pulsed laser-induced stress wave for posttranscriptional gene silencing. *Plant Sci.* **171**, 375–381.
20. Warthmann, N., Chen, H., Ossowski, S., Weigel, D., and Herve, P. (2008) Highly specific gene silencing by artificial miRNAs in rice. *PLoS ONE* **3**, e1829.
21. Gheysen, G. and Vanholme, B. (2007) RNAi from plants to nematodes. *Trends Biotechnol.* **25**, 89–92.
22. Wesley, S. V., Christopher, A. H., Neil, A. S., MingBo, W., Dean, T. R., Qing, L., Paul, S. G., Surinder, P. S., David, A., Peter, A. S., Simon, P. R., Andrew, P. G., Allan, G. G., and Peter, M. W. (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J.* **27**, 581–590.
23. Filichkin, S. A., DiFazio, S. P., Brunner, A. M., Davis, J. M., Yang, Z. K., Kalluri, U. C., Arias, R. S., Etherington, E., Tuskan, G. A., and Strauss, S. H. (2007) Efficiency of gene silencing in *Arabidopsis*: direct inverted repeats vs. transitive RNAi vectors. *Plant Biotechnol. J.* **5**, 615–626.
24. Stephan, O., Rebecca, S., and Detlef, W. (2008) Gene silencing in plants using artificial microRNAs and other small RNAs. *Plant J.* **53**, 674–690.
25. Carole, L. T., Louise, J., David, C. B., and Andrew, J. M. (2001) Size constraints for targeting post-transcriptional gene silencing and for RNA-directed methylation in *Nicotiana benthamiana* using a *potato virus X* vector. *Plant J.* **25**, 417–425.
26. Bleys, A., Vermeersch, L., Van Houdt, H., and Depicker, A. (2006) The frequency and efficiency of endogene suppression by transitive silencing signals is influenced by the length of sequence homology. *Plant Physiol.* **142**, 788–796.
27. Sijen, T., Fleenor, J., Simmer, F., Thijssen, K. L., Parrish, S., Timmons, L., Plasterk, R. H. A., and Fire, A. (2001) On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* **107**, 465–476.
28. Kerschen, A., Napoli, C. A., Jorgensen, R. A., and Müller, A. E. (2004) Effectiveness of RNA interference in transgenic plants. *FEBS Lett.* **566**, 223–228.
29. Kohli, A., Twyman, R. M., Abranches, R., Wegel, E., Stoger, E., and Christou, P. (2003) Transgene integration, organization and interaction in plants. *Plant Mol. Biol.* **52**, 247–258.
30. Small, I. (2007) RNAi for revealing and engineering plant gene functions. *Curr. Opin. Biotechnol.* **18**, 148–153.
31. Senthil-Kumar, M., Hema, R., Ajith, A., Li, K., Udayakumar, M., and Kirankumar, S. M. (2007) A systematic study to determine the extent of gene silencing in *Nicotiana benthamiana* and other Solanaceae species when heterologous gene sequences are used for virus-induced gene silencing. *New Phytol.* **176**, 782–791.
32. Jackson, A. L., Burchard, J., Leake, D., Reynolds, A., Schelter, J., Guo, J., Johnson, J. M., Lim, L., Karpilow, J., Nichols, K., Marshall, W., Khvorova, A., and Linsley, P. S. (2006) Position-specific chemical modification of siRNAs reduces “off-target” transcript silencing. *RNA* **12**, 1197–1205.
33. Van Houdt, H., Bleys, A., and Depicker, A. (2003) RNA target sequences promote spreading of RNA silencing. *Plant Physiol.* **131**, 245–253.
34. Dimitri Semizarov, L. F., Aparna, S., Paul, K., Halbert, D. N., and Fesik, S. W. (2003) Specificity of short interfering RNA determined through gene expression signatures. *Proc. Natl. Acad. Sci. USA* **100**, 6347–6352.
35. Khvorova, A., Reynolds, A., and Jayasena, S. D. (2003) Functional siRNAs and miRNAs exhibit strand bias. *Cell* **115**, 209–216.
36. Muckstein, U., Tafer, H., Hackermüller, J., Bernhart, S. H., Stadler, P. F., and Hofacker, I. L. (2006) Thermodynamics of RNA–RNA binding. *Bioinformatics* **22**, 1177–1182.
37. Birmingham, A., Anderson, E. M., Reynolds, A., Ilsley-Tyree, D., Leake, D., Fedorov, Y., Baskerville, S., Maksimova, E., Robinson, K., Karpilow, J., Marshall, W. S., and Khvorova, A. (2006) 3′ UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nat. Methods* **3**, 199–204.
38. Dharendra, K., Claes, G., and Daniel, F. K. (2006) Validation of RNAi silencing specificity using synthetic genes: salicylic acid-binding protein 2 is required for innate immunity in plants. *Plant J.* **45**, 863–868.
39. Grimm, D., Streetz, K. L., Jopling, C. L., Storm, T. A., Pandey, K., Davis, C. R., Marion, P., Salazar, F., and Kay, M. A. (2006) Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* **441**, 537–541.

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