

Using RNA interference to increase crop yield and decrease pest damage

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Insects pose a huge threat to crop production. Variations in their genomes cause selection to favor those who can survive pesticides and *Bt* crops. Silencing specific genes by RNA interference is a desirable natural solution to this problem. Recent studies have been successful in producing potent silencing effects by using target double-stranded RNAs through an effective vector system. Transgenic plants expressing RNAi vectors as well as dsRNA containing crop sprays have been successful in the laboratory. The commercial product limitation is creating a cost-effective system which is minimally susceptible to insect resistance.

Abbreviations: RNA interference – RNAi, *Bacillus thuringiensis* crops – *Bt* crops, double-stranded RNA – dsRNA, hairpin RNA – hpRNA, small interfering RNA – siRNA, RNA induced silencing complex – RISC, homologous inverted repeats (HIR)

Introduction

Pesticides have been traditionally used on plant crops to prevent crop damage. Once pesticides were discovered to pollute the environment and be harmful to human health, agriculture research began to focus on alternative safer methods. One of the most successful contributions to crop production was the development of transgenic *Bacillus thuringiensis* (*Bt*) crops. *B. thuringiensis* produce toxic proteins which kill insects that feed on crops and decrease damage. However, some insects have evolved to become *Bt*-toxin resistant leading to a need for alternative solutions [1,2].

RNA interference is a natural process which silences specific genes before being translated. RNAi inducers, in the form of transgenic plants or a crop spray, have the potential to effectively silence specific genes [1,2,3••]. Both techniques have been successful in silencing genes in the laboratory, but many more challenges need to be overcome before they are applied in the field. One of the major problems is that with any treatment, pests will eventually develop resistance. Transgenic plants would be cost-effective by producing RNAi inducers throughout a plant's entire life; however, insects with genetic resistance would survive and reproduce [3••]. Alternatively, sprays can deliver different RNAi inducers throughout the lifetime of a plant, constantly silencing different insect genes.

Sprays are impractical though because they do not cover the entire plant and producing large quantities of RNA inducers is not cost-effective at this time [3••]. These factors are important to consider when producing a commercial RNAi product.

Constructing an appropriate vector/expression system and delivery method significantly affects the level of RNA silencing produced. Vectors containing inverted repeats separated by an intron produce double-stranded RNA (dsRNA) or hairpin RNA (hpRNA) which effectively silences genes of interest [4]. Producing dsRNA in RNase III deficient *E. coli* strains and extracting that product to treat plants as an alternative to RNAi transgenic plants has recently been accomplished [5••]. All of these factors have the potential to be collectively applied and effectively silence genes of interest in agriculture. The key to an RNAi commercial product will be targeting genes that potently kill pests or inhibit toxin resistance.

RNAi: A Conserved Mechanism across Different Organisms

RNA interference (RNAi) is a natural process that is highly conserved in many organisms and silences genes by degrading mRNA before it is translated [6]. Particularly, plants and insects

have evolved to use RNAi as protection against viruses [7••,8]. RNA interference silences genes through a process in which a double-stranded RNA (dsRNA) or hairpin RNA (hpRNA) molecule is broken into small interfering RNAs (siRNAs) by an RNase III Dicer. The siRNAs then assemble into an RNA-induced silencing complex (RISC) and guide complementary mRNAs through the RISC where they are degraded [5••]. Recent efforts have been aimed at understanding RNAi mechanisms in different organisms.

Plants possess a natural RNAi mechanism to degrade dsRNAs produced by viruses. However, viruses have evolved a way to inhibit RNAi effects. One way viruses do this is by expressing viral suppressors of RNAi (VSRs). It has been shown that expressing VSRs are an important part of effective viral infection. Viruses have also evolved a way to prevent dsRNAs from going through the RNAi machinery in the host plant, or to halt the degradation of target RNAs. In response to viral evolution, the genes responsible for RNAi in plants have also evolved creating an “arms race” between the two organisms [7••]. This paradigm of evolution is important to consider when developing a commercial product because insects are capable of becoming resistant to RNAi.

Insects have an innate RNAi mechanism which defends insects against viruses [8••]. Recent efforts have been geared towards understanding the RNAi mechanism of insects when they ingest dsRNAs. It was thought that because insects lacked genes encoding RNA-dependent RNA polymerase (RdRP), they were not capable of amplifying siRNAs which causes a systemic RNAi response [9]. Studies hypothesized that this amplification does exist but probably from different genes [1]. Recently, the systemic response has been shown to occur through endocytosis of dsRNAs. However, insects with mutant RNAi components and defective uptake pathways do not experience gene silencing [8••]. This natural phenomenon is important to consider when developing a commercial product because insects with deficient RNAi machinery have the potential of conferring resistance to RNAi plants. Nevertheless, the presence of a systemic response allows efficient gene silencing in insects when they ingest dsRNA.

Efficient Gene Silencing

Various methods for constructing RNAi plants have been successful in the laboratory. Vectors containing inverted repeats have been used to create target dsRNAs which trigger the silencing effect [10]. Figure 1 illustrates a hypothetical RNAi vector and the production of dsRNA for RNAi. The orientation of the inverted repeats depends on the direction in which silencing occurs along the target sequence. The proper orientation will affect gene silencing. Homologous inverted repeats (hIR) and transitive vectors are two variations used in RNAi. Two gene fragments inversely oriented into a vector and separated by an intron create an hIR RNAi vector. A single gene fragment, located upstream of an inverted repeat, creates a transitive RNAi vector. While both hIR and transitive vectors show silencing effects, the hIR vector has proven to be more effective than the latter [6].

Once the RNAi vector has been constructed, stable and efficient replication is desired inside the host. A number of factors which would overcome this challenge have been tested. One factor is promoter strength. Hirai et al. constructed an RNAi vector and compared the standard Cauliflower Mosaic Virus (CaMV) 35S promoter with the replacement of an expression enhancing promoter, the E1 Ω sequence. This experiment showed an increase in gene silencing with the use of the E1 Ω sequence containing vector as opposed to the standard CaMV 35S transcription driven vector [4]. This shows that silencing effects can be enhanced by using specific promoters that enhance transcription. Another factor that creates stable replication is the spacer between the inverted repeats. A spacer length of 1007 bp is most effective in gene silencing compared to 100, 500, and 1800 bp spacers. However, the effect of the length compared to the sequence of the spacer showed that the sequence had more influence on gene silencing than the length [4]. Intron sequences, in particular, have proven to be the best spacers because their non-gene specificity reduces the potential of off-target effects [4,6,11]. While these findings are helpful for future construction of RNAi vectors, careful consideration must be given to the details of the system of interest.

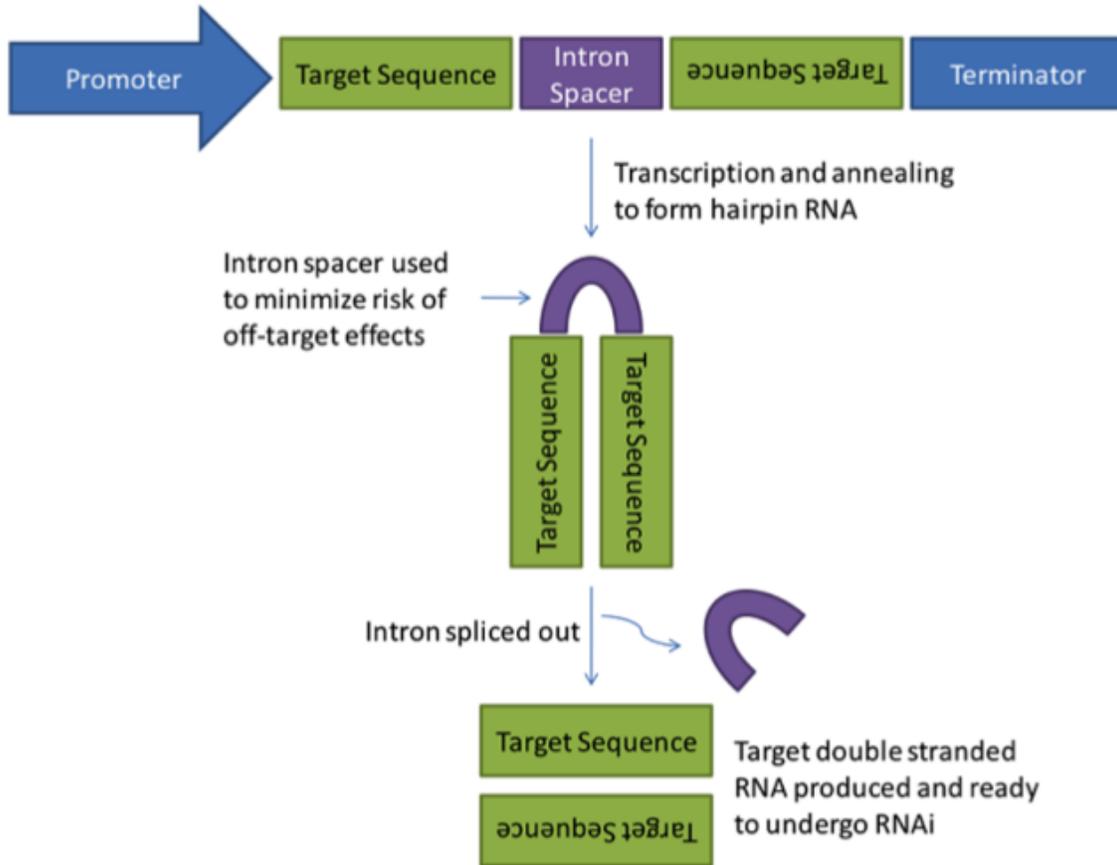


Figure 1. A schematic representation of the production of dsRNA from an RNAi vector. Specific promoter, inverted repeats, and an intron spacer are used to produce the best RNAi effects.

Transgenic RNAi Plants

Transgenic RNAi plants are constructed in the same way other genetically modified plants. First, the target gene of interest must be isolated, amplified, and cloned into a plasmid. This plasmid is then allowed to undergo recombination into an *Agrobacterium tumefaciens* binary vector. Plants are then transformed with *A. tumefaciens* and the vectors replicate to produce dsRNA or hpRNA for RNAi induction [6].

One of the current challenges has been ensuring that the proper inverted repeat can successfully make dsRNA and cause a silencing effect once inside the plant. A current approach to address this problem is constructing an RNAi vector with two partially complementary long oligonucleotides. The two oligonucleotides anneal to produce a partial dsDNA molecule. Multiple cloning steps are unnecessary beyond this point, which is an advantage when developing time-sensitive commercial

products not only because multiple cloning steps are eliminated but also because the inverted repeats are constructed before cloning into the vector. The resulting dsDNA is then cloned into a binary vector that produces two RNAi transcripts [12••]. This method proves that it is possible to transform plants with inverted repeats that can produce stable dsRNAs for RNAi plants.

While laboratory research shows that plants genetically engineered with vectors containing inverted repeats can stably produce dsRNAs, field applications are limited by selective pressure. Insects with resistant genotypes, maybe those possessing genetic variations in their RNAi machinery, will not be affected by RNAi transgenic plants. Those with this defense will eventually multiply and create a new problem for agriculturists. A technique which is able to flexibly change

which target genes it silences is a potential alternative to transgenic plants [3••].

Double-stranded RNA Spray

In 2004, Tenllado et al. showed that a spray consisting of crude preparations of dsRNA were efficient at silencing genes of plant viruses. The dsRNAs were able to stably exist on leaves for several days and confer resistance to viral infection. Even though the Tenllado group was successful in their laboratory research, they recognized that one of the biggest limitations to a cost-effective commercial surface spray is producing mass quantities of dsRNAs [13]. Thus, finding the best vector/host combination which would produce the highest output of dsRNA became a goal in RNAi research.

RNase III cleaves dsRNA and is conserved throughout different organisms, including *Escherichia coli*. *E. coli* can be used as a host to produce a high yield of target dsRNA; however, the presence of the *rnc* gene which produces RNase III may prevent the production of the highest possible dsRNA yield. To solve this problem, a particular strain of *E. coli* which contains a point mutation at the *rnc* gene, and thus lacks of functional RNase III enzyme, is used as a host for the RNAi vector [••5]. This expression system was used in conjunction with a vector that stably expresses dsRNA in *E. coli* [14]. This recent method for producing mass amounts of dsRNA is a positive step forward for future widespread applications.

Aside from successfully discovering a method to produce large quantities of dsRNA, a surface spray is still undesirable because it might not always cover the entire plant from root to leaf. This is especially a problem where larvae live in the soil and feed on roots. When leaves were treated with dsRNA, minimal damages to the leaves were observed; however, root damage still occurred and stunted plant growth [3••]. RNAi enriched soil in conjunction with RNAi spray is a possible solution to this problem. Yet, the cost-effectiveness of these methods is probably still outweighed by the time saving production of RNAi transgenic crops.

Silencing the Right Genes

A number of phenotypic and genotypic changes can be observed in RNAi plants. In the lab, the presence of transgenic RNAi is first confirmed by PCR [15]. Extracting the total mRNA at the end of the procedure is essential to establish a difference

between RNAi treatment and control. In the control, one would expect to see specific mRNAs and not in the insects which fed on the transgenic RNAi plant. Protein levels should also be decreased in the RNAi affected insect since this is a post-transcriptional process [4-6]. The presence or lack of these factors has been a good indicator in determining whether or not laboratory RNAi methods have been effective. However, observing insect mortality and decreased crop damage is what is important in the field.

Killing insects is desired to effectively decrease crop damage. Therefore, essential genes involved in metabolism are key RNAi targets because without these insects cannot survive [3••]. A successful study showed that transgenic plants producing dsRNAs targeting essential ATPase genes were effective at stunting growth and killing the western corn rootworm (WCR) [1]. One attractive notion about essential genes is that they are highly conserved across organisms and so multiple pests could possibly be controlled at once. Targeting essential genes is a promising strategy that could be used in conjunction with *Bt* crops.

Other genes of interest for knock-down are those which cause insects to be resistant to toxins. In addition to *Bt* resistance, insects confer resistance to natural toxins. Gossypol is a compound produced by cotton plants that is toxic to insects. It has been discovered that the cotton bollworm possesses a P450 monooxygenase gene to resist the toxic effects of gossypol. RNAi transgenic plants producing dsRNAs targeting the P450 monooxygenase gene have been successful at protecting plants from cotton bollworm damage [2]. Deducing resistant genes like this in other insects will be helpful in developing the right target dsRNAs.

One of the current approaches in targeting genetic variation is to construct RNAi vectors capable of suppressing multiple genes. As mutations in genes occur and pests survive, new targeting dsRNAs are desired for RNAi. To suppress genes with various homologs, a single transgene can contain partial gene sequences specific to each variation. This recent study also shows that extension of the transgene is possible [16•]. With this in mind, creating transgenic plants targeting multiple variations of genes could greatly reduce insect resistance. In addition, as insect resistance genes are discovered, existing transgenes can be extended to silence those resistance genes as well.

Conclusions

Efficient methods for gene silencing as a means of controlling plant pests have been successfully demonstrated in laboratory settings. RNA silencing effects have been enhanced by the type of vector used to produce dsRNA or hpRNA. Silencing is more potent when inverted repeats of the target sequence are separated by an intron and transcription is driven by a particular promoter. Long oligonucleotides are beneficial in speeding up the cloning process. While these are all important contributions to a commercial product, a concrete system for delivering RNAi inducers is still a challenge.

RNAi transgenic plants and double-stranded RNA containing sprays have both been successful in the laboratory. Transgenic RNAi plants are attractive because they stably produce dsRNA over a long period of time. Whereas a dsRNA spray might be used to target various genes over the lifetime of a crop in response to specific pests. With the ability to construct transgenic RNAi plants capable of si-

lencing multiple variations of genes, the problem of insect resistance can be more cost-effectively solved.

Due to evolution, a one-size-fits all solution to solve insect caused crop damage may never be achieved. Pests will always find a way to resist natural and genetically engineered toxic effects. RNAi is a safe alternative to chemical pesticides because it specifically targets genes in plant pests. In particular, figuring out which insect genes confer resistance will aid in making target dsRNAs. RNAi use in plants has the potential to be used with *Bt* crops and other pesticides in order to decrease damage and increase crop yield.

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Referencesⁱ

1. Baum JA, Bogaert T, Clinton W, Heck GR, Feldmann P, Illagan O, Johnson S, Plaetinck G, Munyikwa T, Pleau M, *et al.* Control of coleopteran insect pests through RNA interference. *Nat Biotechnol* 2007; **25**:1322-1326. [PubMed](#) [doi:10.1038/nbt1359](https://doi.org/10.1038/nbt1359)
 2. Mao YB, Cai W, Wang J, Hong G, Tao X, Wang L, Huang Y, Chen X. Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. *Nat Biotechnol* 2007; **25**:1307-1313. [PubMed](#) [doi:10.1038/nbt1352](https://doi.org/10.1038/nbt1352)
 - 3. Zhao YY, Yang G, Wang-Pruski G, You MS. *Phyllotreta striolata* (Coleoptera: Chrysomelidae): Arginine kinase cloning and RNAi-based pest control. *European Journal of Entomology* 2008; **105**:815-822.
- This is a more recent study which shows that dsRNA sprayed onto plant leaves effectively minimizes insect damage
4. Hirai S, Oka S, Adachi E, Kodama H. The effects of spacer sequences on silencing efficiency of plant RNAi vectors. *Plant Cell Rep* 2007; **26**:651-659. [PubMed](#) [doi:10.1007/s00299-006-0277-4](https://doi.org/10.1007/s00299-006-0277-4)
 - 5. Yin G, Sun Z, Liu N, Zhang L, Song Y, Zhu C, Wen F. Production of double-stranded RNA for interference with TMV infection utilizing a bac-

terial prokaryotic expression system. *Appl Microbiol Biotechnol* 2009; **84**:323-333. [PubMed](#) [doi:10.1007/s00253-009-1967-y](https://doi.org/10.1007/s00253-009-1967-y)

This is a fascinating recent study which describes finding the appropriate vector and host to make dsRNA which makes plants resistant to the Tobacco Mosaic Virus.

6. Filichkin SA, DiFazio SP, Brunner AM, Davis JM, Yang ZK, Kalluri UC, Arias RS, Etherington E, Tuskan GA, Strauss SH. Efficiency of gene silencing in *Arabidopsis*: direct inverted repeats vs. transitive RNAi vectors. *Plant Biotechnol J* 2007; **5**:615-626. [PubMed](#) [doi:10.1111/j.1467-7652.2007.00267.x](https://doi.org/10.1111/j.1467-7652.2007.00267.x)
- 7. Obbard DJ, Gordon KHJ, Buck AH, Jiggins FM. The evolution of RNAi as a defense against viruses and transposable elements. *Philosophical Transactions of the Royal Society B-Biological Sciences* 2009; **364**:99-115. [PubMed](#) [doi:10.1098/rstb.2008.0168](https://doi.org/10.1098/rstb.2008.0168)

This is a comprehensive review of how viruses have evolved to defend themselves against plants' innate RNAi machinery

- 8. Saleh MC, Tassetto M, Rij RP, Goic B, Gausson V, Berry B, Jacquier C, Antoniewski C, Andino R. Antiviral immunity in *Drosophila* requires systemic RNA interference spread. *Nature* 2009; **458**:346-350. [PubMed](#) [doi:10.1038/nature07712](https://doi.org/10.1038/nature07712)

This recent study shows that systemic RNAi effects occur through endocytosis of dsRNA in insects. It also shows that insects can possess defective RNAi machinery which makes them susceptible to viral infection.

9. Price DRG, Gatehouse JA. RNAi-mediated crop protection against insects. *Trends Biotechnol* 2008; **26**:393-400. [PubMed](#) [doi:10.1016/j.tibtech.2008.04.004](https://doi.org/10.1016/j.tibtech.2008.04.004)
10. Tang GL, Galili G, Zhuang X. RNAi and micro-RNA: breakthrough technologies for the improvement of plant nutritional value and metabolic engineering. *Metabolomics* 2007; **3**:357-369. [doi:10.1007/s11306-007-0073-3](https://doi.org/10.1007/s11306-007-0073-3)
11. Luo K, Harding SA, Tsai CJ. A modified T-vector for simplified assembly of hairpin RNAi constructs. *Biotechnol Lett* 2008; **30**:1271-1274. [PubMed](#) [doi:10.1007/s10529-008-9673-x](https://doi.org/10.1007/s10529-008-9673-x)
- 12. Higuchi M, Yoshizumi T, Kuriyama T, Hara H, Akagi C, Shimada H, Matsui M. Simple construction of plant RNAi vectors using long oligonucleotides. *J Plant Res* 2009; **122**:477-482. [PubMed](#) [doi:10.1007/s10265-009-0228-6](https://doi.org/10.1007/s10265-009-0228-6)

This recent study uses long oligonucleotides to eliminate the need for PCR amplification and multiple cloning steps involved in producing RNAi vectors.

13. Tenllado F, Llave C, Diaz-Ruiz JR. RNA interference as a new biotechnological tool for the control of virus diseases in plants. *Virus Res* 2004; **102**:85-96. [PubMed](#) [doi:10.1016/j.virusres.2004.01.019](https://doi.org/10.1016/j.virusres.2004.01.019)
14. Bao S, Cagan R. Fast cloning inverted repeats for RNA interference. *RNA* 2006; **12**:2020-2024. [PubMed](#) [doi:10.1261/rna.258406](https://doi.org/10.1261/rna.258406)
15. Sunilkumar G, Campbell LM, Puckhaber L, Stipanovic RD, Rathore KS. Engineering cottonseed for use in human nutrition by tissue-specific reduction of toxic gossypol. *Proc Natl Acad Sci USA* 2006; **103**:18054-18059. [PubMed](#) [doi:10.1073/pnas.0605389103](https://doi.org/10.1073/pnas.0605389103)
- 16. Hassani-Mehraban A, Brenkman AB, Broek NJF, Goldback R, Kormelink R. RNAi-Mediated Transgenic Tospovirus Resistance Broken by Intraspecies Silencing Suppressor Protein Complementation. *Mol Plant Microbe Interact* 2009; **22**:1250-1257. [PubMed](#) [doi:10.1094/MPMI-22-10-1250](https://doi.org/10.1094/MPMI-22-10-1250)

This study took an already made transgene cassette capable of silencing multiple variations of a gene and extended it to silence one more variation

^{i i} Papers of special significance that have been published within the past five years are highlighted as follows:

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- of special significance