CHAPTER

1

TARGETED GENE SILENCING IN PLANTS USING RNA INTERFERENCE

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0-9664027-8-2/03/$0.00+$.50 *From:* **RNA Interference (RNAi)~Nuts & Bolts of siRNA Technology** (pp.23-36)

Edited by: David Engelke, Ph.D.

1.1 Scope of the Chapter

Cosuppression, post-transcriptional gene silencing (PTGS), and virus-induced gene silencing (VIGS) all describe homology-dependent gene silencing phenomena in plants that involve what is more broadly known as RNA interference (RNAi). The study of these phenomena in plants has provided numerous key discoveries in the field of RNAi research. These include initial evidence of homology-dependent gene silencing by transgenes[1-3], the finding that mobile silencing signal(s) can spread systemically cell-to-cell[4,5], the demonstration (coincident with studies in *C. elegans*) that double-stranded RNA triggers RNAi[6], the discovery of small interfering RNAs (siRNAs) corresponding to eliminated mRNAs[7], and the discovery of RNA-mediated DNA methylation[8,9]. Furthermore, the insight that developmental processes probably make use of RNAi-like mechanisms occurred when subunits of the RNAi machinery in Drosophila were found to correspond to the plant developmental genes ARGONAUTE[10,11] and CARPEL FACTORY[12,13].

In this chapter, we have focused on the design and use of vectors for targeted gene silencing in plants using RNAi, providing only a brief historical perspective of RNAi research in plants. Readers interested in a more extensive discussion of the plant literature can consult a number of recent reviews[14-18].

1.2 A Brief History of RNAi in Plants

The phenomenon of homology-dependent gene silencing leapt onto the scientific stage through serendipity. Two seminal studies were those of Napoli and Jorgensen[2] and Van der Krol *et al.*[3], each attempting to make purple petunias an even darker purple by introducing a transgene designed to overproduce the chalcone synthase enzyme. Much to their surprise, over-expression of chalcone synthase transgenes did not increase coloration. Instead, some flowers were completely colorless (white), and others showed interesting patterns such as loss of pigmentation along the veins but full pigmentation elsewhere. It was the complete loss of color in some or all flower cells that indicated a new phenomenon had been stumbled upon, for not only was the transgene silent in these cells but the endogenous copy of the chalcone synthase gene was silenced, as well. Napoli and Jorgensen thus introduced the term "cosuppression" to describe the coordinate silencing of a transgene and its endogenous homolog(s). Other early studies yielded related findings, such as the unexpected gene silencing that occurred upon introduction of a transgene that shared sequence homology with a pre-existing transgene[1].

Transgenes are commonly introduced into the genomes of most model plant species using *Agrobacterium tumefaciens*, a common soil bacterium that trans-

fers to susceptible plants a section of DNA, called T-DNA (transferred DNA), that is carried on a resident plasmid. Single T-DNAs can integrate into the genome, but it is very common for multiple copies to integrate in variously permuted head-to-head, tail-to-tail and head-to tail arrays. It was soon realized that homology-dependent gene silencing occurred most frequently among transgenes that integrated in multiple copies[19]. Though silencing can occur both transcriptionally and post-transcriptionally, post-transcriptional silencing was found to occur exclusively among tandem transgenes arranged in inverted orientation[20], consistent with the idea that complementary RNAs might somehow be involved.

Plant virologists deserve credit for several major breakthroughs in our mechanistic understanding of RNAi. A number of labs had shown that plant virus resistance could be engineered by expressing viral proteins in transgenic plants, initially using viral coat protein genes[21], but soon recognizing that resistance could be conferred by expression of other viral genes, too[22]. A breakthrough came when Van der Vlugt et al. transformed plants with genes encoding both translatable and non-translatable viral coat protein mRNAs[23]. Importantly, lines expressing non-translatable coat protein mRNA were resistant to infection, indicating that resistance was ocurring at the RNA level and not at the protein level. In related experiments, the Dougherty lab demonstrated that either full length or partial Tobacco Etch Virus sequences could interfere with infection and lead to elimination of corresponding RNAs[24,25]. In a prophetic discussion, Dougherty and colleagues postulated that virus resistance could result from transcription of highly expressed viral RNAs by an RNA dependent RNA polymerase, thus producing antisense copies of the RNAs. They further postulated that resulting RNA duplexes could then be degraded by a specific RNase, thus explaining the elimination of viral RNAs in engineered virus-resistant plants. Well ahead of their time, these predictions have proven to be remarkably accurate.

Many early experiments in plants had demonstrated that over-expression of either sense or antisense RNAs could induce post-transcriptional silencing and did so at similar, relatively low frequencies. Testing the hypothesis that double-stranded RNAs resulting from the production of both sense and antisense RNAs might trigger post-transcriptional gene silencing, Waterhouse *et al.* showed that the frequency of viral or reporter gene silencing was dramatically increased if transgenes were engineered to produce duplex RNAs in the cell[6]. This could be triggered by crossing transgenic lines, one expressing a sense transcript and the other an antisense transcript. Likewise, high frequency silencing was conferred by transgenes that expressed a transcript containing an inverted repeat such that the transcript could fold back on itself to form a double-stranded RNA (dsRNA). These experiments showing that dsRNA triggers RNA elimination in

plants occurred nearly simultaneously with those of Fire et al. that demonstrated that hairpin RNA molecules induce RNA interference in *C. elegans*[26]. These and subsequent findings meshed well with the Dougherty lab's hypothesis that dsRNA molecules initiated the cascade of events leading to silencing[25]. Subsequent biochemical and genetic experiments in *Neurospora*, *Drosophila*, mammals and plants have further supported Dougherty and colleagues' hypothesis. Indeed, the requirement for RNA-dependent RNA polymerase activity in RNAi has been firmly established, as has the involvement of nucleases that cleave the dsRNAs into small RNA molecules. Resulting small RNA fragments may then be targeted for degradation, or may bind to homologous RNAs, priming production of new dsRNAs and amplifying the silencing signal (for an excellent review see[27]).

Greater than ninety percent of all plant viruses are RNA viruses that replicate through a dsRNA intermediate. So, it seems likely that RNA interference in plants has evolved, in part, as a means for protection against viral infection and retrotransposon proliferation[28,29]. Indeed, small interfering RNAs (a hallmark of RNAi) homologous to viral sequence have been found systemically in viral infected plants[30] and mutations in genes involved in RNAi lead to increased susceptibility to disease and to transposon proliferation[31]. Likewise, some plant viruses encode genes which counteract the plant's RNAi surveillance system, thus evading degradation[17,32]. Important for its proposed role in combating disease, RNAi can act systemically throughout a plant. One clear demonstration came from grafting the root and lower portion of a plant in which a reporter gene was silenced by RNAi to a shoot in which the same reporter was active. Amazingly, a silencing signal traversed the graft junction to cause silencing in the shoot[4]. Likewise, infecting a reporter gene-expressing plant with a virus that included sequences matching the reporter gene caused reporter gene silencing far beyond the site of infection[5]. Thus it is thought that cells that initiate RNAi, as in the response to virus infection, can generate small RNAs, or some other mobile signal, which traffic via the phloem throughout the plant[33] and help target related RNAs, thus protecting against a spreading RNA virus.

1.3 Using RNAi for Targeted Gene Silencing in Plants

RNAi has rapidly gained favor as a "reverse genetics" tool to knock down the expression of targeted genes in plants, as in other species, due to certain advantages that RNAi technology holds over gene disruptions caused by transposon or T-DNA insertion. The ability to target multiple gene family members with a single RNAi-inducing transgene is one such advantage. Another is that gene knockdowns due to RNAi are dominant, whereas insertional or other loss-of-function mutations are recessive. The dominant aspect of RNAi allows the

knock down of genes in polyploid genomes that contain four or more orthologs and are thus refractive to traditional mutagenesis[34]. Likewise, orthologs can be knocked down in F1 hybrids in which the RNAi-inducing transgene is introduced through only one of the parents. Finally, the dominance of RNAi allows one to save time by eliminating the additional generations needed to identify individuals that are homozygous for recessive loss-of-function alleles.

Current technology for inducing RNAi in plants derives from the work of Waterhouse and colleagues demonstrating efficient target gene silencing upon expression of an mRNA containing an inverted repeat[6,35]. In some cases, the silencing efficiency of RNAi vectors is reportedly enhanced by the addition of an intron interposed between the inverted flanking target sequences within the RNAi-inducing transgene[36]. However, other groups, including a consortium of labs knocking down chromatin-related genes using RNAi (see http://www.chromdb.org) has found no obvious difference in the effectiveness of dsRNA constructs that use introns or other sequences interposed between the inverted repeats (unpublished). Nevertheless, the Waterhouse design provides the foundation for all currently used plant RNAi-inducing constructs, both for stable transformation and transient transfection.

1.4 Vectors for Stable Transformation

Several families of RNAi vectors that make use of Agrobacterium tumefaciens -mediated delivery into plants have been made available to the public and are being widely used. All share the same overall design, diagrammed in Figure 1-1, but differ in terms of selectable markers, cloning strategies and other elements (see ***Table 1-1***). Two vectors developed by Waterhouse and colleagues at CSIRO in Australia allow for relatively straightforward incorporation of targeting sequences in the sense and antisense direction with an intron sequence between them[37,38]. The first is the pHANNIBAL vector, whose T-DNA (the portion of the plasmid transferred to the plant genome via *Agrobacterium*-mediated transformation) includes a selectable marker gene and a strong promoter upstream of a pair of multiple cloning sites flanking an intron. This structure allows one to clone sense and antisense copies of a gene of interest, separated by the intron. A derivative of the pHANNIBAL vector, pHELLSGATE2, was engineered to facilitate high-throughput cloning of targeting sequences. The efficiency of the pHELLSGATE vector provides a potential advantage for large scale projects seeking to knock down entire categories of genes, including an ongoing effort to knock down the complete set of expressed genes in the Arabidopsis genome (http://www.agrikola.org). Specifically, the pHANNIBAL vector was modified by replacing the polylinkers with aatB site-specific recombination sequences, allowing introduction of targeting sequences of interest using the Gateway technology from Invitrogen (Carlsbad, CA). Briefly, the Gateway sys-

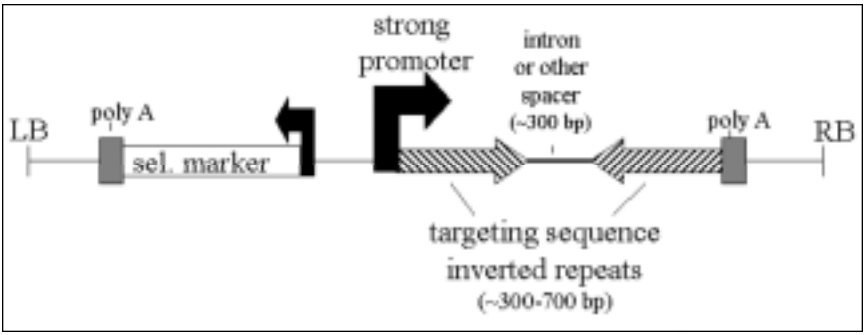

Figure 1-1: Typical organization of a plant RNAi-inducing transgene. The RNAi-inducing transgene consists of a strong promoter driving expression of sequences matching the targeted mRNA(s). These targeting sequences are cloned in both orientations flanking an intervening spacer, which can be an intron or a spacer sequence that will not be spliced. For stable transformation, a selectable marker gene, such as herbicide resistance or antibiotic resistance, driven by a plant promoter, is included adjacent to the RNAi-inducing transgene. The selectable marker gene plays no role in RNAi but allows transformants to be identified by treating seeds, whole plants or cultured cells with herbicide or antibiotic. For transient expression experiments, no selectable marker gene would be necessary. This diagram depicts a T-DNA (transferred DNA) delivered into plant cells by *Agrobacterium tumefaciens*. Thus the T-DNA is flanked by the left border (LB) and right border (RB) sequences that delimit the segment of DNA that is transferred. For stable transformation mediated by means other than *A. tumefaciens*, LB and RB sequences are irrelevant.

tem allows one to use traditional ligation techniques, topoisomerase-mediated cloning or recombination to clone a targeting sequence of interest into a Gateway entry vector plasmid. One's sequence of interest is now flanked by site-specific recombination sites, allowing it to be recombined into the pHELLSGATE2 vector using Invitrogen's proprietary recombinase. Using this technology, one can create a library of gene fragments of interest flanked by recombination sites, and then rapidly and without traditional ligase-mediated cloning, introduce the genes into the pHELLSGATE2 vector. pHELLSGATE8 is identical to pHELLSGATE2 but contains the more efficient aatP recombination sites.

Another set of RNAi vectors for *Arabidopsis* and maize are freely available through the Arabidopsis Biological Resource Center (ABRC, http://www.arabidopsis.org) and were donated by the Functional Genomics of Plant Chromatin consortium (http://www.chromdb.org). Descriptions and complete sequences for these ChromDB RNAi vectors is available at the ChromDB website (http://www.chromdb.org). Briefly, the recommended vectors (pFGC5941 and

pMCG161) include within the T-DNA a selectable marker gene, phosphinothricin acetyl transferase, conferring resistance to the herbicide Basta, and a strong promoter (the 35S promoter of Cauliflower Mosaic Virus) driving expression of the RNAi-inducing dsRNA (see Figure 1-1). Introduction of target sequences into the vector requires two cloning steps, making use of polylinkers flanking a petunia chalcone synthase intron, an overall design similar to pHANNIBAL. Other ChromDB RNAi vectors offer kanamycin or hygromycin resistance as plant selectable markers, instead of Basta resistance, and a non-intronic spacer sequence instead of the chalcone synthase intron. The ChromDB vectors are based on pCAMBIA plasmids, developed by the Center for Application of Molecular Biology to International Agriculture (CAMBIA), which contain two origins of replication, one for replication in *Agrobacterium tumefaciens* and another for replication in *Escherichia coli* (http://www.cambia.org/). Thus, all cloning steps can be conducted in *E. coli* prior to transforming the finished vector into *A. tumefaciens* for plant transformation.

1.5 Design of Targeting Sequences

RNAi vectors are typically designed such that the targeting sequence corresponding to each of the inverted repeats is 300-700 nucleotides in length. Several studies have looked at the minimum size of targeting sequences necessary for RNAi-mediated knockdowns. However, early work focused not on double-stranded sequences but on sense or antisense clones. Transformation of tobacco plants with constructs expressing either 640 or 490 nucleotides of potato virus X movement protein RNA rendered plants fully resistant to the virus. By contrast, four out of eighteen plants expressing a 320 nucleotide sequence were sensitive to the virus and all plants expressing a 140 nucleotide sequence were sensitive[39]. Thomas and colleagues, studying recombinant potato virus X, found that sequences as short as 23 nucleotides could, to some degree, silence a GFP target gene[40]. Interestingly, one clone of 27 nucleotides failed to yield any silencing of GFP. Sequencing of this clone revealed a single PCR-generated error in the middle, separating the sequence into 12 and 14 nucleotide strings of exact homology. The fact that this construct failed to silence GFP indicates that a stretch of perfect complementarity larger than 14 nucleotides is needed.

The exact size of a dsRNA needed to trigger RNAi in plants and other eukaryotes is still not entirely clear. In an in vitro assay using *Drosophila* embryo lysates, a 49bp dsRNA was no more effective at inducing target mRNA degradation than was buffer alone, whereas a 149bp dsRNA decreased target mRNA levels ~ 50%, and 505bp and 957bp dsRNAs decreased target gene mRNA levels by 75-80%[41]. These findings agree with the conventional wisdom among plant researchers using RNAi to generate dsRNAs of 300-700 bp, with a

greater success of silencing correlated with the use of longer sequences. However, a recent study that used DNA microarrays to monitor the genome-wide consequences of RNAi in human cells found reduced levels of mRNAs bearing as little as 11 bp of identity to the targeting dsRNA[42].

1.6 Transient Expression and Other Applications of RNAi Technology

Numerous plant species, including most agricultural crops, are not yet amenable to stable genetic transformation. In this case, functional characterization of genes of interest may be conducted by transiently expressing RNAi-inducing dsRNA transcripts in cells. One simple and popular method for transiently expressing genes in intact tissue is particle bombardment, a method by which DNA is precipitated onto the surface of microscopic gold or tungsten particles and shot into cells at high velocity using a so-called "particle gun"[43]. Using this approach, recent studies in wheat and barley have detailed the effectiveness of bombarding plant tissue with plasmids bearing genes that express dsRNAs, realizing 5-10 fold decreases in target gene expression[44,45].

In addition to its uses in well-studied, but difficult to stably transform species, such as cereals, RNAi has proven useful in plants that are not model experimental systems, including walnut[46] and coffee[47], in the latter case to create decaffeinated coffee plants. RNAi mediated knockdown of genes has also been demonstrated in cultured plant cells, such as rice callus[35].

1.7 RNAi Facilitates New Genetic Studies in Hybrids

The dominant nature of RNAi makes it an important means for knocking down expression of targeted genes in plant hybrids. Classically, the investigation of gene function in hybrids has been problematic. Because the majority of mutations are recessive, out-crossing a recessive mutant in order to form an F1 hybrid results in rescue of the mutation due to the wild-type gene contributed by the second parent, thus masking any phenotype of interest. Though selfing the F1 hybrid to produce F2 individuals homozygous for the mutation might be possible for some species, no two F2 individuals are alike due to segregation and random assortment of all of the other genes from both parents, thus obscuring the unique genetic constitution of the F1 hybrids. The problem is further exacerbated in inter-species hybrids, which are often sterile, such that the F1 provides the only generation that can be studied. For these reasons, RNAi technology is ideally suited for studying the role of particular genes in first generation hybrids, as only one parent needs to carry the RNAi-inducing transgene. Based on these considerations, our lab is exploiting the use of RNAi to explore the role of chromatin modifying genes in stabilizing gene expression patterns and is finding novel phenotypes in newly formed hybrids using this approach.

Acknowledgments

We thank Dr. Richard Jorgensen (University of Arizona) for a pre-print of his book chapter. Research in the Pikaard lab using RNAi methodology is supported by the National Science Foundation under Grant No. 9975930 and by the National Institutes of Health, grant R01-GM60380. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation or National Institutes of Health. S.P. has been supported by the grants listed above and by a National Research Service Award from the National Institutes of Health (1 F32 GM69206).

Table 1-1.

Plant RNAi transformation vectors and their attributes: Vectors pFGC5941 and MCG161 and similar vectors are available from the Plant Functional Genomics of Chromatin Consortium (http://www.chromdb.org) whereas the pHannibal and pHellsgate vectors are available from CSIRO[37]. Inserts are cloned into pFGC5941, pMCG161 and pHannibal using standard restriction digest and ligation protocols whereas inserts are cloned into pHellsgate using the Invitrogen Gateway system. Double stranded RNA (dsRNA) transcripts are driven by the Cauliflower Mosaic Virus 35S promoter. pFGC5941 contains the petunia chalcone synthase intron interposed between the cloned inverted repeats; pMCG161 contains the rice waxy intron, and pHannibal and pHellsgate contain the *Flavaria trinervia* pyruvate orthophosphate dikinase intron.

	pFGC5941	pMCG161	pHannibal	pHellsgate
Target Organism	dicots	monocots	dicots	dicots
Cloning Method	restriction digest / ligation	restriction digest / ligation	restriction digest/ligation	Gateway recombination
Bacterial Selection	kanamycin	chloramphenicol	spectinomycin	spectinomycin
Plant Selection	Basta	Basta	chloramphenicol	chloramphenicol
dsRNA promoter	CaMV 35S	CaMV 35S	CaMV 35S	CaMV 35S
inverted repeat spacer	ChsA intron	Waxy intron	Pdk intron	Pdk intron
Reference	chromdb.org	chromdb.org	ref #37	ref #37

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RNAi/small RNA-based gene silencing is widely used as a popular means to study gene function because it can target specific genes of known sequences to decipher their functions for the first time in a non-random manner. It can lead to gene silencing at either the transcriptional (target DNA methylation) or post-transcriptional levels (target RNA cleavage or translational repression)232425.Â Upon virus infection, plant defence system triggers RNA interference (RNAi) pathway which is a homologydependent gene silencing mechanism implicating degradation of complementary RNA. Employing small RNA molecules such as miRNA and siRNA, RNAi has been widely manipulated to engineer resistance in plants against viruses.