# RNAi for functional genomics in plants

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## Abstract

RNAi refers to several different types of gene silencing mediated by small, dsRNA molecules. Over the course of 20 years, the scientific understanding of RNAi has developed from the initial observation of unexpected expression patterns to a sophisticated understanding of a multi-faceted, evolutionarily conserved network of mechanisms that regulate gene expression in many organisms. It has also been developed as a genetic tool that can be exploited in a wide range of species. Because transgene-induced RNAi has been effective at silencing one or more genes in a wide range of plants, this technology also bears potential as a powerful functional genomics tool across the plant kingdom. Transgene-induced RNAi has indeed been shown to be an effective mechanism for silencing many genes in many organisms, but the results from multiple projects which attempted to exploit RNAi on a genome-wide scale suggest that there is a great deal of variation in the silencing efficacy between transgenic events, silencing targets and silencing-induced phenotype. The results from these projects indicate several important variables that should be considered in experimental design prior to the initiation of functional genomics efforts based on RNAi silencing. In recent years, alternative strategies have been developed for targeted gene silencing, and a combination of approaches may also enhance the use of targeted gene silencing for functional genomics.

***Keywords:*** *RNAi; gene silencing; plants; genomics; maize; gene expression*

## DISCOVERY OF RNA-MEDIATED SILENCING

RNA-induced gene silencing (RNAi), was originally observed as unusual expression patterns of a transgene designed to induce overexpression of chalcone synthase in petunia plants [1]. Shortly after this initial observation, similar results were reported by other researchers in a range of organisms ([2–4], reviewed by ref. [5]). In the years following these observations, experiments in many model systems contributed to rapid advancements in understanding the underlying mechanisms, and RNA-mediated gene silencing processes came to be collectively known as RNA interference (RNAi). It was learned that the 'triggers' for RNAi were small RNAs, 21–25 nts in length, that were processed from longer, double-stranded (ds) RNAs by endonuclease proteins referred to as dicers [6–9]. These siRNAs direct degradation of mRNAs in a homology dependent manner, leading to post-transcriptional silencing of the silencing target, or direct heterochromatin formation and DNA methylation at regulatory sequences for the target to be silenced, thus inducing transcriptional silencing of target loci in a homology dependent fashion (reviewed by ref. [10]). Now, it is understood that RNAi is an evolutionarily conserved mechanism for gene regulation that is critical for many examples of growth and development.

## TRANSGENE-INDUCED RNAi

Nearly as soon as the mechanisms underlying RNAi were uncovered, researchers began devising creative applications to exploit these mechanisms. The discovery of RNAi coincided with expansion of public sector use of genetic transformation in plants during the 1990s, and a natural product of that coincidence was the use of transgenes to generate dsRNAs that would trigger silencing of endogenous genes in a homology dependent fashion. The efficacy of this approach in plants was first described in the late 1990s [11], and it was demonstrated that transgenes that include a segment of gene sequence in an inverted repeat orientation around a spacer

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region will generate a dsRNA when expressed in plants. This dsRNA is processed into siRNAs that can trigger silencing in a homology-dependent manner. Since the initial report of its efficacy, transgene induced RNAi has been used to silence many endogenous target genes, resulting in metabolically engineered plants with improved storage capacity, virus resistance, oil content, and health benefits (reviewed by ref. [12]).

## DIVERSE PATHWAYS OF RNA SILENCING

There are multiple pathways by which small RNA molecules can influence gene expression in plants, at both the transcriptional and post-transcriptional levels. These pathways vary in their sources of small RNAs and specific mechanisms of silencing [10, 13, 14]. Accordingly, transgene-derived dsRNAs can be used to trigger silencing via multiple pathways. If the trigger sequence bears homology to the coding region of a target gene, post-transcriptional silencing is typically initiated, resulting in degradation of the target gene mRNA. If the trigger sequence bears homology to the promoter of the target gene, transcriptional silencing is typically initiated.

## ADVANTAGES OF siRNA-INDUCED SILENCING FOR GENOMIC APPLICATIONS

Because transgene-induced RNAi has been effective at silencing one or more genes in a wide range of plants, this technology also bears potential as a powerful functional genomics tool across the plant kingdom. A common strategy for functional genomics projects is to generate lines that are deficient for the activity of a subset of genes, and test the knock down lines for phenotypes to characterize the function of the knocked down gene. In many cases, assessing a phenotype requires the presence of specific alleles of marker genes, necessitating several generations of crosses to test a specific mutant allele for such a genotype. Using transgene-induced RNAi, a single copy of the inducing transgene is frequently sufficient to induce silencing, which simplifies many assays that might be required to assay phenotypes in a functional genomics project. Additionally, inclusion of a selectable marker gene can facilitate at least the initial stages of genotyping, since the presence and activity of at least a portion of the transgene will be evident by the expression of the selectable marker. Another potential benefit of this technique is the ability to silence multiple target genes at once, and circumvent genetic complementation of silencing events by expression of close homologs in the genome. In many cases, a single inverted repeat transgene can be designed to silence multiple, closely related genes [15]. To accomplish this task using point mutations or insertional mutations might require multiple generations of crosses to generate genetic stocks that are double or triple mutants for a specific gene family. For an essential gene, inducible silencing constructs can be used to generate the equivalent of conditional mutants [16]. In this case, a transgene with an inverted repeat that silences the essential gene constitutively might be lethal, but the use of an inducible promoter will allow an individual which bears the transgene to survive, and also to express the transgene and silence the gene of interest under specific conditions so that the resulting phenotype can be studied.

## TECHNIQUES FOR INDUCING dsRNA-MEDIATED SILENCING IN PLANTS

RNA-mediated silencing can be induced at the transcriptional and post-transcriptional levels, using a variety of sources of dsRNA (reviewed by ref. [13]). In many cases, the level of silencing is not experimentally determined, but is assumed to occur at the transcriptional or post-transcriptional level depending upon the sequence that is included in the dsRNA-generating transgene. To induce transcriptional silencing with a transgene, a typical strategy involves designing a construct such that a dsRNA is generated which bears homology to the promoter region of the intended silencing target [17]. The siRNAs trigger downstream effectors that induce cytosine methylation and other epigenetic modifications at the promoter region of the target gene, resulting in transcriptional inhibition of the gene. This type of silencing is thought to result in heritable silencing that will persist in the absence of the inducing transgene, and is likely mediated by the RNAi-mediated DNA methylation and heterochromatin formation pathway (reviewed by ref. [14]). Herein, this method of silencing will be referred to as promoter directed RNA silencing.

To induce post-transcriptional silencing with a transgene, a portion of the coding region of the gene is typically introduced into an inverted repeat (IR) construct, and expression of that transgene will result in a dsRNA with homology to the coding region of the intended silencing target [18]. This type of silencing is likely mediated by components of the *trans*-acting siRNA pathway in plants (reviewed by ref. [14]). Herein, this method of silencing will be referred to as coding region directed RNA silencing.

## VARIABILITY IN SILENCING OF TARGET GENES

RNAi has indeed been shown to be an effective mechanism for silencing many genes in many organisms, including several agriculturally significant plants (Table 1), but the results from projects which utilized RNAi on a genome-wide scale suggest that there is a great deal of variation in the silencing efficacy between transgenic events, silencing targets and silencing-induced phenotypes [15, 19–23].

In almost every plant species for which transgene-induced RNAi has been used to silence a target gene, some variability in the extent of silencing has been reported. In Arabidopsis, transgene induced RNAi was used to target silencing of ~25 endogenous genes [19]. Multiple transgenic lines were observed for each RNAi-inducing construct, where the constructs were designed to induce coding region directed RNA silencing. There was some variability in silencing efficiency between lines bearing the same construct, but results were largely consistent if only single copy, T4 generation transformants were compared for a given construct. Notably, a subset of target genes were not observed to be silenced in any of the lines that were assayed. Similarly, in maize, [15], attempts to target a subset of genes resulted in variable silencing between silencing lines for specific constructs. For these experiments, the constructs were also designed to induced coding region directed RNA silencing. In some cases, a transgenic event would trigger silencing in the generations immediately following transformation and outcrossing, but not in the T2 or later generations. One potential reason for this is the transcriptional silencing of the RNAi-inducing transgene itself, which would prevent the inducing dsRNAs from being generated to trigger silencing. Alternatively, some genes may be resistant to silencing by this technique for other, as of yet undetermined reasons. As in Arabidopsis, a subset of the maize genes assayed for silencing appeared to be resistant to silencing using this particular approach. Similar variability in level of knock down, as measured at the RNA level, has been reported by numerous individuals using transgene-induced RNAi in plants [23, 24].

Variability has also been reported at the phenotypic level, meaning that the expected phenotype for silencing a given gene can vary in the degree of phenotype expressed, or if the phenotype is expressed at all. In maize, silencing of a large number of chromatin-related genes via coding region directed RNA silencing [15] resulted in relatively few notable phenotypes (McGinnis *et al.*, unpublished data). Transgene induced RNAi has been used to alter flower color in gentian plants, but there was variation between the extent of pigmentation between different transgenic clones [25]. In wheat, comparisons of many different experiments indicated that anywhere from 33 to 100% of transgenic events resulted in plants exhibiting the expected phenotypes for silencing of the target gene (reviewed by ref. [21]). In soybean, silencing was demonstrated for lipoxygenase genes at the molecular level, but the anticipated nodule phenotypes were not observed [26]. Low levels of silencing could indicate that the silenced genes were redundant with other genes which retained normal expression in the transgene-induced RNAi line. Alternatively, these observations could be explained by a failure to silence the target gene in all tissues of the plant, as silencing was only directly measured by RT-PCR using RNA from a few tissues. In this case, the phenotype could be dependent upon expression of the target gene in one or few specific tissues, and the transgene may not be inducing silencing of the target in this tissue. Yet another explanation could be that although expression of the target gene mRNA is reduced, a small amount of transcript remains and is sufficient to maintain wild type functionality.

Each of these examples represents efforts to induce post-transcriptional gene silencing, but similar variability has been observed for attempts to induce transcriptional silencing of endogenous gene targets (Table 1). For example, in rice, siRNA-inducing transgenes were generated to target promoters of endogenous genes for transcriptional silencing. The target genes did exhibit cytosine methylation, but transcriptional silencing did not occur [27].

**Table 1:** Summary of transgene induced-dsRNA silencing in some agriculturally significant plants

| Plant | Silencing target | Stable or transient transformation | Promoter or coding region targeted? | Percentage of transgenic plants exhibiting silencing | Method of assaying silencing | Reference |
|---|---|---|---|---|---|---|
| Tobacco | Polyphenol oxidase | Stable | Coding region | 70% | Phenotype | [39] |
| Cotton | Δ12-desaturase | Stable | Coding region | 80% | Phenotype | [39] |
| Cotton | Δ9-desaturase | Stable | Coding region | 60% | Phenotype | [39] |
| Cotton | Δ12-desaturase | Stable | Coding region | 53% | Phenotype, northern blot | [40] |
| Cotton | Δ9-desaturase | Stable | Coding region | 62% | Phenotype, northern blot | [40] |
| Cotton | Myb transcription factor | Stable | Coding region | Not reported[d] | Phenotype, RT-PCR | [41] |
| Coffee Bean | CaMXMT1 | Stable | Coding region | Not reported[d] | Phenotype, RT-PCR | [42] |
| Oilseed rape | B-type MADS-box | Stable | Coding region | 22.6% [a] | Phenotype, in situ | [43] |
| Opium poppy | Codeine reductase | Stable | Coding region | 100%[b] | Phenotype, RT-PCR, northern blot | [44] |
| Tomato | DET1 | Stable | Coding region | Not reported[d] | Phenotype, RT PCR | [45] |
| Tobacco | Chalcone isomerase | Stable | Coding region | 100%[b] | Phenotype, northern blot | [46] |
| Tomato | ACC oxidase | Stable | Coding region | 87 and 27%[c] | Phenotype, northern blot | [47] |
| Soybean | 24 kDa Oleosin A | Stable | Coding region | Not reported[d] | Phenotype, immunoblotting | [48] |
| Canola | farnesyltransferase | Stable | Coding region | Not reported[d] | Northern blot | [49] |
| Potato | Rar1 (required for Mla12 resistance) | Transient | Coding region | Not reported[d] | RT-PCR | [34] |
| Wheat | Zinc finger, CCT domain protein | Stable | Coding region | Not reported[d] | RT-PCR, phenotype | [50] |
| Wheat | MADS, K-box domain protein | Stable | Coding region | Not reported[d] | RT-PCR, phenotype | [51] |
| Wheat | Starch branching enzyme IIa | Stable | Coding region | 92% | Immunoblotting, phenotype | [52] |
| Wheat | Starch branching enzyme IIb | Stable | Coding region | 33% | Immunoblotting | [52] |
| Wheat | Transmembrane protein | Stable | Coding region | 33% | RT-PCR | [53] |
| Wheat | Phytoene desaturase | Stable | Coding region | 78% | RT-PCR | [53] |
| Wheat | NAM/NAC transcription factor | Stable | Coding region | 29% | RT-PCR | [54] |
| Wheat | Seed storage protein | Stable | Coding region | 100% | RT-PCR, SDS–PAGE | [55] |
| Barley | GAMyb transcription factor | Transient | Coding region | Not reported[d] | Phenotype | [56] |
| Barley | Slender protein | Transient | Coding region | Not reported[d] | Phenotype | [56] |
| Barley | ABA-inducible kinase | Transient | Coding region | Not reported[d] | Phenotype | [56] |
| Rice | Heme oxygenase | Stable | Promoter | Not reported[d] | RT-PCR | [30] |
| Rice | RAC GTPase 1 | Stable | Promoter | 0% | RT-PCR | [30] |
| Rice | RAC GTPase 3 | Stable | Promoter | 0% | RT-PCR | [30] |
| Rice | RAC GTPase 4 | Stable | Promoter | 0% | RT-PCR | [30] |
| Rice | Putative PolyA Binding protein | Stable | Promoter | 0% | RT-PCR | [30] |
| Rice | Putative CBS domain protein | Stable | Promoter | 0% | RT-PCR | [30] |
| Rice | Putative ribosomal protein L5 | Stable | Promoter | 0% | RT-PCR | [30] |
| Maize | Male Sterility Factor 45 | Stable | Promoter | 78–90%[c] | Phenotype, RT-PCR | [28] |
| Maize | dihydrol flavenol reductase | Stable | Promoter | Not reported[d] | Phenotype | [28] |
| Maize | cytochrome P450 | Stable | Promoter | Not reported[d] | Phenotype | [28] |
| Potato | Granule bound starch synthase I | Stable | Coding region | 48–87%[c] | Phenotype, northern blot | [57] |
| Potato | Granule bound starch synthase I | Stable | Promoter and Coding region | 50% | Phenotype, northern blot | [29] |
| Potato | Granule bound starch synthase I | Stable | Promoter | 5–60%[c] | Phenotype, northern blot | [29] |

For each report, efficiency was calculated as the percent of plants reported to exhibit silencing.
[a]Silencing was reported to be unstable and not heritable.
[b]Level of silencing was reported to be variable, but silencing was observed in all transgenic plants.
[c]Separate values reported for silencing induced by distinct constructs.
[d]Percentage of plants exhibiting silencing not reported, but silencing was reported for some plants.

Variability of promoter-directed silencing has been reported in other plants [28–30], suggesting that this technique may not be any more consistent than coding region-directed silencing. In fact, the most consistent levels of silencing induced with transgene-induced dsRNAs have been observed for the silencing of trangenes rather than endogenous loci, leading to the speculation that this type of silencing may not be efficient for silencing endogenes [10].

There are many factors that could, and likely do, contribute to the variability of silencing at the molecular and phenotypic levels using RNAi-induced techniques. These factors should be considered when designing experiments that are dependent upon the successful use of these techniques.

## EXPERIMENTAL DESIGN CONSIDERATIONS

In spite of the inherent limitations associated with transgene induced RNAi, it is still a potentially useful tool for functional genomics applications. However, results from prior projects indicate that several important variables should be considered in experimental design prior to the initiation of reverse functional genomics efforts based on RNAi-induced silencing. To generate an effective silencing line for a gene of interest, it is important to consider each of the caveats and limitations discussed above. First, the transgene construct intended to trigger silencing should provide reliable expression throughout many stages of development, and be stably expressed in the host plant. This is of particular importance for post-transcriptional silencing experiments, where the dsRNAs need to be present in the same tissue as the target mRNA for silencing to occur. Additionally, transgene expression should be evaluated as soon as possible for each event, and over multiple generations to insure that each line is stably-silencing its target. Many transgenic events should be generated and analyzed, so that lines with active transgenes that are effectively inducing silencing of their intended targets can be selected for and maintained. Where possible, it would be advantageous to assay for expected phenotypes as well, so that useful lines can be identified and propagated. In some cases, multiple silencing strategies may have to be attempted for reverse genetics purposes, when the goal is to identify a phenotype for a gene of interest.

## ALTERNATIVE STRATEGIES

In recent years, alternative strategies have been developed for targeted gene silencing, and a combination of approaches may also enhance the manipulation of gene silencing for functional genomics. In Arabidopsis, techniques have been developed to screen expression libraries via RNAi silencing to identify silencing targets which generate a phenotype of interest [31]. This may be an efficient alternative to a one gene at a time approach for some experiments, and this alternative also eliminates any bias based on pre-selection of silencing targets. In many organisms, transient assays have been used to identify genes that are susceptible to silencing and for which silencing results in the phenotype of interest [32–34]. This may be a useful technique to narrow-down targets and optimize silencing protocols, and thus increase the likelihood of success for some projects. Additionally, there are alternative methods that can be utilized to induce silencing, which may be useful in cases where a given target seems resistant to silencing by one method. Artificial miRNAs [35], virus-induced gene silencing [36], and zinc finger nuclease mediated-genome modifications [37, 38] have all been developed for modifying gene expression in plants. Each of these are potential alternatives for silencing genes that are recalcitrant to silencing by other techniques.

**Key Points**

- RNAi is a recently discovered, biologically significant type of gene regulation.
- In recent years, RNAi has been increasingly adapted and exploited to manipulate gene expression in a range of species.
- Results from these projects indicate a high degree of variability in using this technique.
- Potential sources of this variability should be considered in experimental design for future and ongoing projects.

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