现代生物技术前沿

RNAi:
A Guide to Gene Silencing

〔美〕G. J. 汉农 主编

RNAi
——基因沉默指南

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科学出版社
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图字 01-2004-0172

内 容 简 介

RNA组学研究日益蓬勃发展。RNA的相关研究被美国《科学》杂志评为2002年的十大进展。人们发现，双链RNA可以抑制含有特定序列的基因表达。应用RNA的这一特点，在生物体外强有力地使基因沉默——即RNA干扰（RNAi），从而开创了一个新的和正在快速发展的生命科研领域。RNA干扰技术可以加速人们对人类基因功能的认识，而且科研人员有望利用这种技术设计出使致病基因失活的新型基因药物。

本书由这一领域内的著名专家撰写，内容新颖全面，系统性强，具有很高的参考价值。主要内容包括RNA干扰的基本概念、生物化学研究，双链RNA对基因组的调节，哺乳动物基因表达中的shRNA介导沉默，核酶超家族，以及RNA干扰在多种动物模型中使基因沉默的方案。

本书适合发育生物学、细胞生物学、遗传学、基因组学、神经生物学和分子生物学等生命科学相关领域的教学研究人员以及本科生、研究生参考。

书名原文：RNAi：A Guide to Gene Silencing
原著正文内彩图集中列于书末彩色图版，请读者对应参考。

图书在版编目（CIP）数据

RNAi：基因沉默指南/（美）汉农（Hannon，G. J.）主编. —影印本. —北京：科学出版社，2004.1
（现代生物技术前沿）
ISBN 7-03-012685-8

Ⅰ. R… Ⅱ. 汉… Ⅲ. 核糖核酸-生物技术-英文 Ⅳ. Q522

中国版本图书馆CIP数据核字（2003）第122979号

责任编辑：莫结胜
责任印制：刘士平/封面设计：王 浩

科学出版社 出版
北京东黄城根北街16号
邮政编码：100717
http://www.sciencep.com
深海印刷有限责任公司印刷
科学出版社发行 各地新华书店经销
*
2004年1月第 一 版 开本：890×1240 1/16
2004年1月第一次印刷 印张：27 3/4 插页：8
印数：1—3 000 字数：923 000

定价：65.00元

（如有印装质量问题，我社负责调换〈环伟〉）

Preface

My first exposure to RNA interference (RNAi) came at a Pew Scholars meeting in Puerta Vallarta, Mexico in 1998. Craig Mello gave a short, informal talk about a remarkable observation—exposing *Caenorhabditis elegans* to double-stranded RNA (dsRNA) caused potent and sequence-specific silencing of homologous genes. Beyond this, the response to dsRNA had a number of nearly unbelievable properties. Silencing occurred not only in cells directly exposed to the dsRNA, but also spread throughout the organism and into its progeny. We were all confounded and amazed by this seemingly inexplicable set of observations.

I am fairly certain that, at the time, Craig had not yet realized the extent to which his observations would take hold of biology to create an exciting new field, which at the time of the writing of this book, seems to be expanding at an exponential rate. Shortly after the original report of RNAi, Rich Carthew, another Pew Scholar, extended many of Andrew Fire's and Mello's basic observations about the ability of dsRNA to suppress gene expression into *Drosophila* embryos. Biochemical and genetic studies led to the realization of a common silencing response that extended throughout eukaryotes from plants (where the phenomena were really first noted) to fungi to animals.

Since its inception, the study of RNAi has proceeded along two parallel tracks. The first is an effort to understand the biology of this response—its mechanism and its role in the organism. The second has been the desire to develop RNAi as an experimental and possibly therapeutic tool. This duality has made the planning and construction of this book a challenge. In the end, it was decided that the volume should provide both an introduction to the biology of RNAi and practical advice, including detailed protocols, on its application in numerous systems.

The work described herein represents a field that is moving at an astonishing pace. Therefore, I am all the more grateful to the authors, who took time from their very busy schedules to contribute to this volume. This book would also not have been possible without the constant support (read nagging and prodding) of the people at Cold Spring Harbor Laboratory Press, namely, Mary Cozza, Judy Cuddihy, David Crotty, and John Inglis. I am also indebted to those colleagues in the field who have provided encouragement and support of our work over the past several years. I am especially thankful for the talented group of graduate students and postdoctoral fellows including Scott Hammon, Emily Bernstein, Yvette Seger, Amy Caudy, Ahmert Denli, Jian Du, Izabela Sujka, Jose Silva, Patrick Paddison, Doug Conklin, and Michelle Carmell. I am grateful to my family, Gretchen, Will, and Claire, for their support and tolerance of the time that this endeavor has consumed.

Greg Hannon

Contents

Introduction

Gregory J. Hannon

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THE FIELD OF INQUIRY THAT FOCUSES ON RNA INTERFERENCE (RNAi) was not born out of the desire to answer a specific biological question. Instead, it coalesced around an observation by Craig Mello and Andrew Fire that double-stranded RNA (dsRNA) could induce a sequence-specific silencing response in *Caenorhabditis elegans*. This observation led immediately to the hypothesis that a set of homology-dependent silencing mechanisms might be related as responses to a common silencing trigger, namely, dsRNA. This hypothesis could certainly explain virus-induced gene silencing and some types of transgene cosuppression, particularly those caused by the presence of complex transgene arrays. However, it was (and remains) less clear how other types of homology-dependent silencing responses, for example, copy-number-dependent silencing of unlinked transgenes, might trigger a generic RNAi pathway.

As the field has moved forward, other types of homology-dependent regulatory mechanisms have been drawn into the fold. For example, small temporal RNAs, discovered approximately 10 years ago, are now known to regulate their protein-coding targets via the RNAi machinery. The small temporal RNAs are now recognized as the founding members of a large class of similarly structured noncoding RNAs, most with unknown cellular roles. Maintenance of facultative heterochromatin at the fission yeast centromere requires RNAi, drawing a parallel between dsRNA-mediated phenomena in fungi and the previously demonstrated ability of dsRNA to induce heritable silencing in plants by triggering alterations in chromatin structure. The notion that dsRNA could induce epigenetic change via the RNAi machinery by acting on the genome was cited as the 2002 "Breakthrough of the Year" by *Science*. However, it is not yet clear to what extent the ever-widening net of RNAi will also be cast over other epigenetic phenomena to include, for example, imprinting and X-chromosome inactivation and epigenetic memory during development.

During the past 5 years, tremendous progress has been made in lifting the veil that hid the underlying mechanism of RNAi and related silencing responses. The molecular underpinnings of the basic aspects of the silencing process have emerged from a combination of biochemical and genetic studies in several model systems. However, as is reflected in the content of this volume, we have really only reached the first level in our understanding of how dsRNA directs the suppression of homologous genes. Specifically, we have identified a number of the participants in the silencing process. However, the precise mechanisms by which they function remain unknown. For example, it is now clear that short interfering RNAs (siRNAs) direct the silencing machinery to its targets, but we have little understanding of how the transcriptome—or for that matter perhaps the genome—is effectively searched. We do not even know precisely which proteins con-

tact the siRNAs within the RNA-induced silencing complex (RISC) or which components of this complex represent its catalytic and functional core.

We do have a basic understanding of how siRNAs are produced, as this seems to occur through the action of a single enzyme, Dicer. However, we do not yet know how Dicer might be regulated, how it recognizes dsRNA substrates with different structures, nor how it might facilitate the incorporation of siRNAs into effector complexes.

The identification of components of the RNAi machinery has led to a number of significant advances. Through the use of genetics, a number of remarkable biological functions for this gene-silencing pathway have been uncovered. One of the key functions of the RNAi machinery in plants was foreshadowed by the observation that infection of a plant with an RNA virus that included a fragment of an endogenous gene caused silencing of that gene. It is now clear that RNAi viruses are combated to a large degree in plants by posttranscriptional gene silencing (PTGS; the plant nomenclature for the RNAi response). Notably, many plant viruses encode proteins that antagonize PTGS, and these proteins are essential for viral replication. Such virulence determinants become dispensable in plants harboring mutations that compromise PTGS, creating a beautiful interlock of genetic experiments that argue strongly for the importance of PTGS as the major antiviral defense in plants.

Genetics studies of RNAi in *C. elegans* have also revealed a role for RNAi in the control of endogenous parasitic nucleic acids. A distinct subset of RNAi-resistant mutants release transposons from repression, creating a mutator phenotype. Perhaps not unrelated are roles in managing repetitive elements at the *Schizosaccharomyces pombe* centromere, where an intact RNAi machinery is essential for formation of heterochromatin, which is in turn required for proper centromere function. In *Tetrahymena*, preliminary evidence, namely, the presence of small RNAs and the requirement for an Argonaute family member, suggests that this organism's strategy for managing repetitive elements also depends on an RNAi-like mechanism. Transposon-like sequences are eliminated from the transcriptionally active macronucleus of *Tetrahymena* in a process that seems to be determined by small RNAs that guide histone methylation.

Considered together, evidence that has emerged over the past few years has perhaps suggested an ancestral function for RNAi in controlling parasitic and pathogenic nucleic acids. This function may have evolved into a more general mechanism for managing repetitive elements. However, it has also become clear that the RNAi machinery is intimately involved in the regulation of endogenous, protein-coding genes, particularly those that control development and possibly also stem cell maintenance. This conclusion grew largely out of the observed phenotypes of Dicer mutants, which connected RNAi to previously characterized small RNAs, the stRNAs, that regulate developmental timing. Since that time, many similarly structured, endogenous nonconding RNAs have joined this family, which are now known collectively as microRNAs or miRNAs. Presently, the functions of almost all of these are unknown.

In addition to a wealth of biological insight, mechanistic studies of RNAi have revealed how to exploit the dsRNA response as a tool for experimental biology with ever-increasing effectiveness. Tools based on RNAi have in some cases begun to revolutionize experimental biology. Ironically, in many ways, the discovery of RNAi itself grew out of attempts to design an approach to performing reverse genetics in *C. elegans*, specifically the use of antisense RNA. Now the phenomenon has come full circle, having taken experimentalists working in numerous model systems by storm and with RNAi largely supplanting the use of antisense technologies by experimental biologists. Of course, the use of RNAi has been *de rigueur* in *C. elegans* for several years, but more recently, the use of dsRNA as a silencing tool has been extended into traditional genetic models such as *Drosophila*, into nontraditional models such as trypanosomes, and into mammalian systems.

With *C. elegans*, the development of genome-wide libraries of RNAi inducers is now permitting investigators to identify nearly every gene required for a specific biological phenotype in a rapid and efficient fashion. Similar libraries are on the horizon for *Drosophila*, trypanosomes, and mammals. Although these libraries will never usurp the position of traditional genetics, they offer a revolutionary capability in somewhat genetically intractable models such as mammalian cells. Furthermore, in many experimental systems, including *C. elegans* and mice, RNAi complements traditional genetic technologies by offering a much more rapid and cost-effective way to mimic the effects of hypomorphic mutations in living animals.

Given the rapid pace of advance in this field, it is difficult to predict a future that will still be the future by the time this volume is published. However, given recent advances in the use of RNAi to engineer heritable silencing in mammals, to alter stem cells for organ reconstitution, and to alter the course of disease in model systems, the transition of RNAi from a tool that permits the analysis and modeling of disease to one that provides a therapy for disease must be anticipated. Although significant hurdles remain to be overcome, the course to therapeutic application of RNAi has already been mapped out by proof-of-principle studies in the literature. These include notable successes in blocking viral replication in cultured cells, as well as studies with HIV and with poliovirus. In the former case, a combination of in vitro studies with the demonstrated ability to stably engineer hematopoietic stem cells may provide the means to immunize cells against HIV infection via autologous transplantation. In vivo, RNAi has been shown to antagonize the effects of hepatitis C virus in mice.

In addition to its use as a therapeutic tool, the RNAi machinery itself may be altered in human disease. Recently, mechanistic studies have revealed potential connections between the Fragile X syndrome and the RNAi machinery. More broadly, genetic and biochemical analyses in *Drosophila* and more recently zebrafish have suggested potential roles for the RNAi machinery in the localization and storage of mRNAs, which can be released upon some stimulus (e.g., neuronal stimulation or fertilization of an egg) to permit regulated protein synthesis. Finally, a consideration of genetic data from plants and *Drosophila* and expression data from mammals suggests a central role for the RNAi machinery in maintenance of stem cell identity. Given all of these suggestions, it seems likely that not only will RNAi be harnessed as a therapeutic tool, but also the RNAi machinery itself may become a therapeutic target.

The extent to which studies of the RNAi machinery have infiltrated biology as a whole is reflected in the diversity of chapters in this volume. This book contains many chapters that are devoted to the application of RNAi as a tool in numerous biological systems. These have been designed as practical guides, discussing both the strengths and weaknesses of using RNAi in a given setting and providing detailed protocols. A number of chapters that illustrate the diversity of silencing processes that are triggered by dsRNA and introduce their biological relevance have also been included. Additional general background is provided in key areas that are essential to understanding RNAi, such as epigenetics and the enzymology of the ribonuclease III family. Given this balanced organization, it is hoped that this volume can provide a starting point to those interested in using RNAi as a tool while also providing a strong background in the biology of this gene silencing response.

CHAPTER 1

Sense Cosuppression in Plants: Past, Present, and Future

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COSUPPRESSION IS DEFINED OPERATIONALLY as the simultaneous reduction in expression of a transgene and homologous, endogenous genes, in which the cosuppressed state is identified by comparison with an opposite state of coexpression of both transgene and homologous endogenes (Napoli et al. 1990; van der Krol et al. 1990). Typically, the coexpression state is observed either in a different physiological or developmental context from the cosuppressed state or as an epigenetic revertant of the cosuppressed state. Because the definition of cosuppression refers to an outcome (*not* to a mechanism), it may be applied correctly to both transcriptional and posttranscriptional silencing (although it has been most commonly used to refer to posttranscriptional silencing).

It was recognized from the outset that cosuppression is phenomenologically distinct from paramutation (and paramutation-like examples of transgene silencing), but that the mechanisms underlying these two phenomena might nevertheless be related (Napoli et al. 1990). Paramutation is the imposition of an epigenetic silencing state on a gene by another silent allele (or homologous gene) that persists after segregation of the causative allele (or gene). Because the paramutagenic (i.e., causative) gene is silent prior to the interaction, the interacting alleles or genes are never observed to be coexpressed, and so cannot properly be referred to as cosuppressed in the silent state. Generally, the cosuppression state is completely reversible for all interacting genes after segregation or epigenetic reversion, whereas paramutation, by definition, can be maintained without the continuing presence of the causative allele in sexual progeny, although perhaps only in some progeny or only for short times.

Over time, the term "cosuppression" came to be used inappropriately to refer to almost any example of transgene silencing in plants, including many examples in which no corresponding coexpression state was demonstrated, and so, through misuse, it fell into disuse. It came to be replaced in the plant literature by more mechanistic terms, such as transcriptional gene silencing (TGS) and posttranscriptional gene silencing (PTGS), both of which could encompass interactions between homologous genes that result in either cosuppression or paramutation, as well as other examples of silencing not even dependent on gene duplication. PTGS now seems to be giving way to the term "RNA silencing," which is more accurate, because the primary target for silencing is a gene's transcript rather than the gene itself (i.e., the gene remains fully active at the transcriptional level). The original meaning of the term cosuppression was revived, and applied in animals, in an influential review by Birchler et al. (1999), and as a result, cosuppression is now widely used to refer to RNA silencing of transposable elements in animals.

Many recent reviews address RNA silencing in plants (Hammond et al. 2001; Matzke et al. 2001; Vance and Vaucheret 2001; Vaucheret et al. 2001; Voinnet 2001; Waterhouse et al. 2001). To not merely reiterate the content of these excellent reviews in this chapter, the principal goals here are to (1) address the nature, mechanism, and implications of "sense cosuppression," a type of cosuppression that is caused by a sense transgene engineered for overexpression of an endogenous protein; (2) provide an historical context for the data and concepts that are relevant to understanding sense cosuppression; and (3) consider how sense cosuppression might have a role in normal posttranscriptional control of supracellular patterns of gene expression and information processing in plants. The important distinction between sense cosuppression and RNA interference (RNAi) for the purposes of this chapter is that the latter is triggered by double-stranded RNA (dsRNA) molecules injected into a cell or produced by transcription of inversely repeated DNA sequences in the genome, whereas the former is initiated by a cellular RNA-directed RNA polymerase (RdRP) that uses sense transcripts as templates to produce the dsRNA molecules that trigger RNA silencing.

EARLY CONCEPTS FOR MECHANISMS OF HOMOLOGY-BASED GENE SILENCING IN PLANTS

At least three broad classes of hypotheses were proposed to explain cosuppression in the first years after its discovery:

- A sensitive response to exceeding some threshold of transcript production (van der Krol et al. 1990; Lindbo et al. 1993; Meins and Kunz 1994).
- Unintended production of antisense RNA, e.g., by readthrough transcription from neighboring promoters (Grierson et al. 1991; Mol et al. 1991).
- Homology-based ectopic pairing causing a perturbation in chromatin state or nuclear localization and resulting in altered transcript fate or transcription rate (Jorgensen 1990; Napoli et al. 1990).

For a detailed review of the early data and concepts, see Jorgensen (1992). Because no single hypothesis seemed able to account for all the data, many participants in the field believed that multiple mechanisms were likely to exist. In time, it came to be believed that promoter homology-based silencing, which is transcriptional, was most likely based on an ectopic pairing (DNA:DNA interaction) mechanism, whereas transcript homology-based silencing, which is posttranscriptional, involved either aberrant transcripts or a threshold response to excessive accumulation of transcripts (for review, see Matzke and Matzke 1995). However, arguments persisted for a DNA pairing-initiated process resulting in a cytoplasmic posttranscriptional state (Jorgensen 1992; Flavell 1994; Baulcombe and English 1996; Que et al. 1997; Stam et al. 1998).

The early data on homology-based gene silencing in plants were based on a variety of types of transgene constructs introduced into different plant species. These diverse experimental systems often produced apparently conflicting results, making it very difficult to draw general conclusions. Thus, it became important to focus on model systems. Three plant species led to significant progress in understanding the complexity of RNA-silencing phenomenology and mechanisms in plants. Due to its long history as an excellent model system in both plant virology and transgenesis, tobacco (and some of its wild relatives) has been particularly useful for RNA-silencing experiments involving viruses, as well as for investigating systemic movement of RNA-silencing signals. *Arabidopsis* has been especially useful for genetic analysis, in particular, the identification of genes necessary for RNA silencing, and will be the key model system for most future efforts.

Petunia had a significant early role because it offered a convenient visible phenotype, flower pigmentation, which was useful for defining different modes of silencing and detecting and monitoring infrequent epigenetic events. Results from each system are described here to the extent that they relate to sense cosuppression.

COPY RNA AND THE INVOLVEMENT OF AN RNA-DEPENDENT RNA POLYMERASE

A major conceptual breakthrough came from the recognition by plant virologists that sense transgenes expressing RNA homologous to a viral genome conferred resistance to that virus, even if the transcript produced no protein product, i.e., the transgene-induced resistance was RNA-mediated (van der Vlugt et al. 1992; Lindbo et al. 1993). Measurement of the transcription rates of different transgenes showed that the highest rates occurred in plants that were resistant to homologous viruses, leading to the conclusion that RNA-mediated resistance might be a threshold phenomenon (Lindbo et al. 1993; Smith et al. 1994; Mueller et al. 1995; English et al. 1996; Goodwin et al. 1996).

To explain threshold-dependent, RNA-mediated silencing, Dougherty and colleagues proposed involvement of a plant-encoded RdRP that would copy a small segment or segments of an RNA that had accumulated to unacceptably elevated levels. These small RNAs would then hybridize with the target RNA, rendering the RNA nonfunctional, and RNases would target the partially double-stranded messenger or viral RNA complex for degradation. A system mediated by RNA has appeal in its relative simplicity, specificity, and the limited amount of genetic information required (Lindbo et al. 1993).

Dougherty and Parks (1995) noted that gene specificity could be provided by RNA molecules as short as 18 bp or so, a suggestion that hit remarkably close to the mark: Guide RNAs that direct RNA silencing via the RNA-induced silencing complex (RISC) are now known to be approximately 21–22 nucleotides long. The proposed involvement of an RdRP enzyme has received strong support from the subsequent discovery that genes homologous to a tomato RdRP are required for RNA silencing in plants, animals, and fungi, although it still remains to be shown whether the proteins encoded by these genes have RdRP activity. In addition, the involvement of a dsRNase, known as Dicer, has now been demonstrated. The evidence for guide RNAs, RdRP homologs, and dsRNases has been reviewed by Hannon (2002) and elsewhere in this volume.

From an historical perspective, it is interesting to note that the first suggestion that a dsRNase might be involved in homology-based gene silencing was actually made by Cameron and Jennings (1991), after observing silencing of one transgene by a homologous transgene in cultured animal cells. These authors suggested that at high concentrations, short complementary regions of sense RNA molecules that normally pair intramolecularly might participate in intermolecular interactions, creating aggregates that might be subject to recognition by a dsRNase.

TWO MODES OF INDUCTION OF RNA SILENCING BY SENSE OVEREXPRESSION TRANSGENES

Recognition that sense transgenes can cause two distinct modes of RNA silencing came from analyses of the frequencies and patterns of chalcone synthase silencing triggered by different types of transgene constructs in petunia flowers (Que et al. 1997). First, silencing of chalcone synthase genes occurred at strikingly different efficiencies, depending on the nature of the transgene construct: A high frequency of silencing (80% of trans-

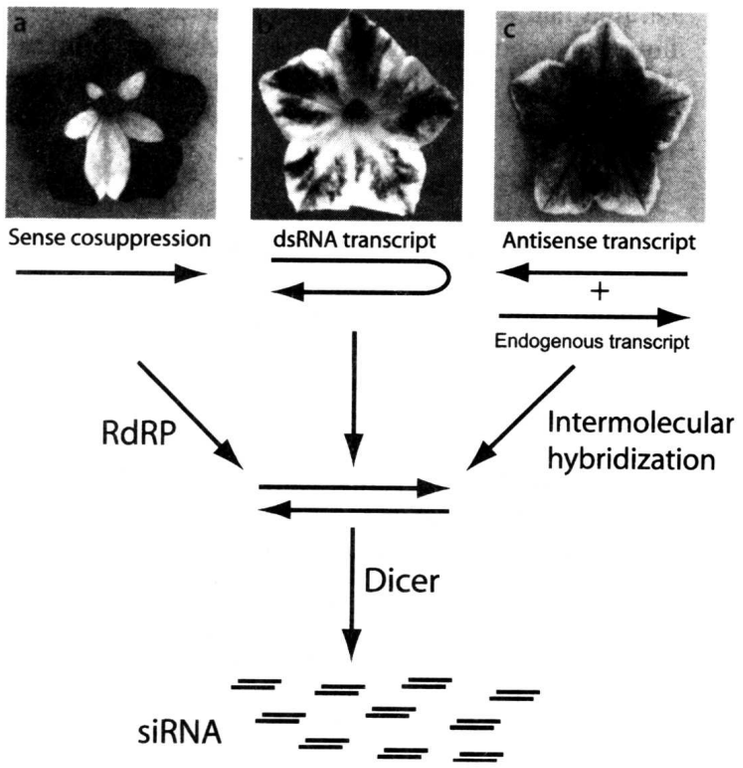

FIGURE 1.1. Modes of dsRNA-mediated silencing of chalcone synthase in petunia induced by (*a*) sense overexpression transgene, (*b*) inverted repeat transgene, and (*c*) antisense transgene. (*a* and *c* reprinted, with permission, from Que et al. 1998 © Blackwell Science.)

genotes) was obtained with constructs engineered for high-level overexpression of chalcone synthase protein (Napoli et al. 1990; Jorgensen et al. 1996), whereas a low frequency of silencing (5–15%) was observed with sense constructs not designed for protein overexpression (van der Krol et al. 1990; van Blokland et al. 1994). The former type of construct also resulted in a greater diversity of phenotypes, ranging from highly ordered, morphology-based, flower color patterns to disordered complex patterns. Single-copy or dispersed transgenes were associated with ordered patterns, whereas inverted repeat transgenes (a very common arrangement of *Agrobacterium*-transferred DNA [T-DNA] molecules) were associated with complex, disordered patterns (Jorgensen et al. 1996; Que et al. 1997). Examples of the patterns produced by these two types of sense transgenes are shown in Figure 1.1 (and are also contrasted with the pattern of silencing that is typical of an antisense construct). Importantly, constructs that were not engineered for protein overexpression caused RNA silencing *only* when the transgene was integrated into the genome as an inverted repeat (Que et al. 1997; Stam et al. 1997).

Two modes of induction of RNA silencing by sense transgenes were thus proposed: one initiated only by sense overexpression transgenes (sense cosuppression) and the other initiated by inverted repeat integrants, regardless of how the transgene was engineered (Que et al. 1997). Further experimental support for the existence of these two modes has been provided by Dalmay et al. (2000b) and Beclin et al. (2002), as discussed in a review by Vance and Vaucheret (2001). Distinct features of the two modes of silencing that can be induced by sense overexpression transgenes are summarized in Table 1.1 and are discussed below.

TABLE 1.1. *Two modes of RNA silencing induced by sense expression transgenes in plants*

1. dsRNA Transcript-mediated Silencing
   - Requires inverted repeat integrant.
   - Does not require strong promoter.
   - Premature nonsense codons do not block silencing.
   - Does not require RNA-dependent RNA polymerase homolog (in *Arabidopsis*).
2. Sense Cosuppression
   - Requires strong promoter driving sense transcript.
   - Single-copy transgene is sufficient.
   - Premature nonsense codons reduce silencing dramatically.
   - Requires RNA-dependent RNA polymerase homolog (in *Arabidopsis*).

dsRNA Transcripts as Triggers of RNA Silencing

The first indication that dsRNA might be a trigger of RNA silencing in plants came from experiments by Waterhouse et al. (1998), who reported that plants carrying both a sense and an antisense transgene homologous to a viral genome were much more resistant to that virus than were plants carrying either a sense or an antisense transgene alone. Hypothesizing that this might be a reflection of involvement of dsRNA in triggering RNA silencing, Waterhouse et al. engineered transgenes with transcribed inverted repeats homologous to the target RNA to produce dsRNA by intramolecular base pairing and found that such constructs were much more efficient inducers of RNA silencing than were sense or antisense transgenes; such constructs producing self-complementary transcripts are now widely used for functional genomics in plants. These experiments immediately suggested that plant transgenes that had integrated as inverted repeats and that were exhibiting RNA silencing were likely to be doing so by means of dsRNA produced by readthrough transcription from one T-DNA copy into another.

The independent parallel discoveries of dsRNA as a trigger of RNA silencing in plants and dsRNA as the trigger of RNAi in worms (Fire et al. 1998) suggested the possibility of a common underlying mechanism. Within a few years, it was demonstrated that plants, animals, and fungi all required several similar genes for dsRNA-mediated silencing (including an RdRP homolog), proving the existence of a common underlying mechanism (for review, see Hammond et al. 2001). Elucidation of the molecular basis of dsRNA-mediated degradation of homologous transcripts in animals, plants, and fungi is progressing rapidly, as can be seen throughout this volume, and it is broadly accepted that dsRNA is the ultimate trigger for this process, even if not the initial trigger in cases such as sense cosuppression.

How Do Single-copy Sense Transgenes Act as Triggers of RNA Silencing?

Key features of a transgene construct that determine whether a single-copy sense transgene can produce RNA silencing in plants are (1) a strong promoter and (2) the absence of a premature nonsense codon in the protein-coding sequence (Que et al. 1997). This suggests that a high concentration of translatable sense transcript must be produced for a single-copy transgene to trigger cosuppression.

It was proposed that single-copy sense transgenes only cause RNA silencing when they are integrated in such a way that antisense transcripts are produced by readthrough from adjacent promoters (Grierson et al. 1991; Mol et al. 1991; Fire and Montgomery 1998). This hypothesis seems inconsistent with the fact that nearly all single-copy trans-

gene integrants are able to trigger RNA silencing if the transgene construct was engineered for overexpression (Jorgensen et al. 1996). In addition, introduction of an early nonsense codon to the coding sequence drastically reduces the ability of single-copy transgenes to trigger silencing (Que et al. 1997). It seems unlikely that a change in several base pairs at a single location in a 1.1-kb transcript (to introduce a nonsense codon) would affect the ability of an antisense readthrough transcript to form dsRNA and trigger silencing, unless one accepts that a slightly lower accumulation of an early nonsense transcript (i.e., the "threshold" hypothesis) could be responsible for its failure to cause silencing. Additional observations inconsistent with a frequent role for antisense readthrough transcripts in sense cosuppression come from a series of constructs expected to be subject to readthrough antisense transcription into chalcone synthase from an adjacent selective marker (Que and Jorgensen 1998). No increase in silencing was observed, contrary to expectation in the antisense readthrough hypothesis.

More direct support for the conclusion that single-copy sense transgenes produce a distinct mode of silencing comes from the demonstration that a single-copy antisense transgene (which carries multiple nonsense codons in its transcript) does not produce the ordered pattern of silencing produced by an allelic sense transgene created by Cre/*lox*-mediated inversion of the coding sequence (Que et al. 1998). Instead, single-copy antisense transgenes reduce pigmentation quantitatively throughout the corolla of the flower (Figure 1.1c). Presumably, the antisense transcript acts by pairing with the endogenous sense transcript to interfere with its translation and/or to produce dsRNA leading to RNA degradation. Finally, removal of nonsense codons from an antisense chalcone synthase transgene produces patterns characteristic of sense transgenes (superimposed on the typical antisense pattern), indicating that an antisense transcript has the capacity to trigger sense cosuppression if translation is not terminated by early nonsense codons (N. Doetsch and R. Jorgensen, unpubl.).

Once a role for dsRNA was suggested by the experiments of Waterhouse et al. (1998), the RdRP that had been proposed by Dougherty and colleagues to copy sense transcripts into cRNA molecules came to be viewed as the source of dsRNA needed for RNA silencing. From this perspective, recognition of a sense transcript to serve as a template for the RdRP enzyme would be the initial event in the sense cosuppression mode of silencing. An RdRP homolog in *Arabidopsis* (SDE1/SGS2) was shown to be required for (certain examples of) transgene-induced RNA silencing, but not for RNA virus-induced RNA silencing (Dalmay et al. 2000b; Mourrain et al. 2000). The likely interpretation of these observations is that because viruses replicate via a dsRNA intermediate produced by a virus-encoded RdRP enzyme, a plant-encoded RdRP is not needed to trigger RNA silencing of replicating RNA viruses, whereas in the case of silencing that is initiated by a sense transgene, it is.

Two important features of RNA silencing in plants are (1) the ability to maintain silencing after removal of the source of the initiator RNA (Lindbo et al. 1993; Palauqui and Vaucheret 1998; Ruiz et al. 1998; Voinnet et al. 1998) and (2) spreading of RNA targeting from the region homologous to the initiator RNA to adjacent regions, both 5′ and 3′ to the region of homology (Voinnet et al. 1998). Investigation of the relationship between maintenance and spreading in *Arabidopsis* showed both processes to be dependent on transcription of the target gene, as well as on the presence of functional SDE1/SGS2, suggesting that maintenance involves production of dsRNA by SDE1/SGS2 using the entire length of the target RNA as template (Dalmay et al. 2000b; Vaistij et al. 2002). Thus, the role of RdRP in sense cosuppression appears to be twofold: (1) to initiate silencing by converting sense transcripts to dsRNA substrates for the DICER nuclease and (2) to produce cRNA molecules for incorporation into the RISC complex for targeting homologous transcripts.

Complications for Interpretation of the Plant Literature

Given the existence of two modes of RNA silencing in transgenic plants, it is clear that complications may arise when a transgene is engineered for overexpression and is then integrated in the plant genome as an inverted repeat. Thus, inverted repeat transgene complexes can trigger both modes of RNA silencing simultaneously, as well as alternately during the development of a plant: For example, some branches produce flower color patterns typical of single-copy chalcone synthase overexpression transgenes, whereas other branches of the plant produce flowers with no silencing or with complex patterns typical of those produced by inverted repeat transgenes driven by weak promoters (Que et al. 1997). In addition, inverted repeats are subject to DNA methylation in plants, and this can result in partial or complete transcriptional silencing of the sense overexpression transgene and/or other promoters in the T-DNA that may be responsible for dsRNA production by readthrough expression. Epigenetic changes in patterns of DNA methylation that occur in inverted repeat integrants can lead to alternate transgene states: For instance, one in which the promoter is methylated, and another in which the transcribed sequences are methylated, but not the promoter (Stam et al. 1998).

Another example of alternate epigenetic states would be a plant in which one state expresses the selective marker transgene while the adjacent sense overexpression transgene has been subjected to transcriptional silencing, and the reciprocal state exhibits transcriptional silencing of the selective marker transgene concomitant with high-level transcription of the sense overexpression transgene. A possible consequence of such alternation of epigenetic states in an inverted repeat integrant is (1) transcriptional dsRNA production via readthrough transcription from the marker transgene into the overexpression transgene in one epigenetic state of the inverted repeat transgene complex and (2) high-level production of sense transcripts from the overexpression transgene in the other state potentially triggering sense cosuppression. In the case of chalcone synthase in petunia flowers, these two distinct modes of silencing can be monitored by observing the patterns of silencing which distinguish the two modes (shown in Figure 1.1). Frequently, however, the two phenotypes are superimposed in the same flower (Jorgensen et al. 1996), indicating simultaneous or coincident occurrence of both modes not only in the same plant, but even in the same organ.

Clearly then, if two distinct modes of initiating RNA silencing can occur in the same plant alternately or simultaneously, interpretation of the results is likely to be compromised. Before this was understood, nearly the entire literature on RNA silencing in plants was based on transgenes engineered in such a way that single-copy integrants were unable to cause RNA silencing, whereas inverted repeat integrants could trigger silencing, whether by sense cosuppression or by dsRNA production via readthrough transcription. In retrospect, analyses of inverted repeat integrants carrying sense transgenes were likely a prime cause of the difficulty of fitting all observations to a single model for the mechanism of RNA silencing. Fortunately, the solution to this problem is relatively simple, although infrequently adopted: Either one should analyze single-copy integrants of sense transgenes designed for protein overexpression (taking care to avoid unnecessary readthrough from neighboring genes, such as selective marker transgenes; see, e.g., Que et al. 1997; Que and Jorgensen 1998), or one should analyze transgenes designed to produce dsRNA transcriptionally via self-complementary transcripts in the manner of Waterhouse et al. (1998).

Keeping in mind the caveat that much of the plant literature is based on inverted repeat integrants of sense transgenes, there are nonetheless interesting observations in that literature relevant to the phenomenon of sense cosuppression, such as a possible role for translation in the initiation of silencing and for DNA methylation in maintaining or propagating silencing states.